VIRUS DISEASES OF *Trifolium subterraneum* AND *Vicia faba* IN TASMANIA

by

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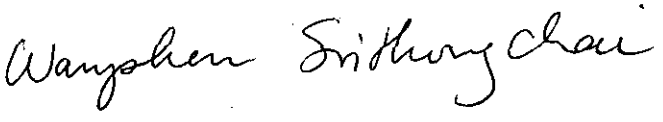
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person, except where due reference is made in the text of this thesis.



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ABSTRACT

Studies were conducted on six virus isolates recovered either from subterranean clover (*Trifolium subterraneum*) or broad bean (*Vicia faba*) infected in the field in Tasmania. Two were identified as isolates of bean yellow mosaic potyvirus (BYMV-F, BYMV-K), two as isolates of clover yellow vein potyvirus (CYVV-B, CYVV-D) and two as isolates of soybean dwarf luteovirus (SDV-Ap, SDV-As). These isolates were compared with respect to some of their biological and physical properties and representatives of the isolates were also studied in the field to compare their spread and effects in plots of broad bean and how that might be controlled with insecticides.

The CYVV isolates generally caused more severe symptoms than the BYMV isolates on those host plants that they had in common. These two groups could be distinguished on the basis of their host ranges but neither host range nor serological tests could differentiate the isolates within the same group. However isolates of the same group did differ with respect to their transmissibility by various aphid species. The differences in the efficiency with which the isolates were transmitted by various aphid species seemed to reflect differences in the helper factors encoded by them. For example *Rhopalosiphum padi* transmitted BYMV-F quite efficiently but it only transmitted CYVV-D if it had prior access to plants infected with BYMV-F.

The host ranges of the SDV isolates were broadly similar although SDV-Ap generally had a more restricted host range and caused milder symptoms on most of the hosts that they had in common. An important difference in host range from an epidemiological

viewpoint was that SDV-Ap infected lucerne *(Medicago sativa)* but not white clover *(Trifolium repens)* while SDV-As infected white clover but not lucerne. This finding was particularly relevant because lucerne is an important oversummering and overwintering host for *Acyrthosiphon pisum*, as white clover is for *Aulacorthum solani*. The two different vector specific isolates (SDV-Ap and SDV-As) could not be distinguished serologically, unlike the barley yellow dwarf virus where serological specificity is closely related to vector specificity. However the viral groups were similar in that transcapsidation was indicated when the two SDV isolates were present together as a mixed infection because then, although *A.pisum* never transmitted SDV-As when this isolate was present by itself, this species did transmit the SDV-As genotype when it was present in plants that were also infected with SDV-Ap.

Field investigations were undertaken over three seasons at Cambridge in south-eastern Tasmania to study the epidemiology and control of some of the virus isolates referred to previously. Infection foci of different virus isolates were established at the centres of plots of broad beans and the subsequent development of virus epidemics was monitored and related to aphid infestation of the plants in the plots and aphid flight activity. Spread of all the viruses was least in plots sown in the late autumn (April or May) and greatest in those sown in the early spring (September). In all three seasons, BYMV spread more rapidly than any of the other viruses. The patterns of BYMV spread were always contagious, radiating from infection foci established in the centres of the plots. The non-colonising *R.padi* as alatae, was implicated as the prime vector of BYMV in the first two seasons while apterous *A.*

pisum colonising the plants were responsible for most of the spread of this virus in the third season. The differences noted in patterns and modes of spread of BYMV between seasons was partly related to plant density in the plots and the nature of neighbouring crops. When compared, the rates of spread of BYMV-F and BYMV-K were similar.

Spread of SDV-Ap in the plots was always greater than that of SDV-As and this was correlated with the relative intensities of aphid flight activity of *A. pisum* and *A. solani* in south-eastern Tasmania. The development of SDV infections in the field plots occurred independently of the infection foci established at the centres of the plots. The distributions of infections of these isolates was non-random, infected plants being concentrated near the boundaries of the experimental area indicating that most SDV infections were established by viruliferous alatae alighting on the experimental area after flying in from external reservoirs of infection, presumably mostly infected lucerne plants.

Experiments were conducted to assess control of secondary spread of the viruses with demeton-S-methyl and of primary and secondary spread with deltamethrin. These experiments were largely unsuccessful because they only provided significant control when infection rates were low (i.e. in the plots sown in late autumn) and pesticide treatments were not warranted for control of virus spread in those sowings.

The BYMV and SDV isolates reduced the yields of broad bean plants in the field. They decreased the numbers of pods per plant, the numbers of beans per pod and the individual bean weights. The effects of all the isolates on yield and its components were greater the earlier the infections were initiated.

The detrimental effects on individual plants were greater for the SDV isolates than the BYMV isolates. However on a crop basis, BYMV was more important in causing yield losses due to its prevalence in the plots.

Selected subterranean clover pastures in drier areas of the State were surveyed regularly for infection with viruses. In addition, some legume crops in the north-western region of Tasmania were examined on a few occasions. The incidence of infection with different viruses was extremely variable between seasons and the incidence of infection with different viruses at the same site and time was never correlated. There were even large differences between levels of infection with a particular virus in pastures that were situated close to one another. The one consistent feature arising from the survey was the relatively common occurrence of SDV-As in the north-west region where white clover is widespread. Infection with SDV transmitted by *A. pisum* was not identified in that region and this contrasted with the situation in south-eastern Tasmania.

The factors determining the occurrence of viruses in pasture legumes and legume crops in Tasmania were concluded to be very complex. A wide array of environmental factors have the potential to affect the interactions between the various viruses, their host plants and their aphid vectors. Many factors that can have a role in affecting virus incidence were elucidated during the course of the field surveys, the field experiments and the greenhouse studies. However it seems unlikely that it would be possible to develop models that could reliably predict either serious virus disease outbreaks or freedom from them. The cropping and farming systems of Tasmania are diverse and intermingled and the climate is mild,

the occurrence of extreme conditions, leading to high or low aphid activity and virus incidence, do not occur.

TABLE OF CONTENTS

		PAGE
TITLE		i
DECLARATION		ii
ABSTRACT		iii
TABLE OF CONTENTS		viii
ACKNOWLEDGEMENTS		xii
CHAPTER 1	**LITERATURE REVIEW**	1
1.1	Introduction	1
1.2	Cultivation of *Trifolium subterraneum* and *Vicia faba*	1
1.3	Characterisation of viruses infecting *Trifolium subterraneum* and *Vicia faba*	5
	Cucumber mosaic virus	10
	Potyviruses	14
	Soybean dwarf virus (syn. subterranean clover red leaf virus)	21
	Subterranean clover stunt virus	28
1.4	Dependent transmission of viruses by aphids	31
	Helper factors associated with the dependent transmission of non-persistent viruses by aphids	31
	Luteoviruses dependent on other luteoviruses for transmission by aphids	34

CHAPTER 2	**GENERAL EXPERIMENTAL PROCEDURES**	40
2.1	Virus isolates	40
2.2	Plants	40
2.3	Aphids	47
2.4	Mechanical inoculation	53
2.5	Electron microscopy	53
2.6	Serological tests	54
CHAPTER 3	**FIELD SURVEY OF VIRUSES IN *Trifolium subterraneum*, *Vicia faba* AND OTHER LEGUME CROPS IN TASMANIA**	57
3.1	Introduction	57
3.2	Materials and Methods	58
	Subterranean clover pastures	58
	Survey of legume crops in north-western Tasmania	60
3.3	Results	61
	Subterranean clover pastures	61
	Survey of legume crops in north-western Tasmania	65
3.4	Discussion	68
CHAPTER 4	**BIOLOGICAL PROPERTIES OF SELECTED POTYVIRUSES AND LUTEOVIRUSES**	70
4.1	Introduction	70
4.2	Materials and Methods	71
	Virus isolates	71
	Differential hosts	71
	Aphid transmission	72
	Dependent transmission of viruses by aphids	74
4.3	Results	75
	Host ranges and symptoms	75

	Aphid transmission	81
	Dependent transmission of plant viruses by aphids	87
4.4	Discussion	93
CHAPTER 5	**PHYSICAL PROPERTIES OF SELECTED POTYVIRUSES AND LUTEOVIRUSES**	97
5.1	Introduction	97
5.2	Materials and Methods	99
	Virus sources and propagations	99
	Purification procedures	100
	Antisera production	105
	Serological tests	105
5.3	Results	107
	Properties of purified viruses	107
	Serological tests	110
5.4	Discussion	121
CHAPTER 6	**FIELD STUDIES ON THE EPIDEMIOLOGY AND CONTROL OF APHID-BORNE VIRUSES IN *Vicia faba***	124
6.1	Introduction	124
6.2	Materials and Methods	125
6.3	1985-1986 Field Studies	126
	Materials and Methods	126
	Results	126
	Recovery of pea aphid specific isolates of soybean dwarf virus	131
6.4	1986-1987 Field Studies	133
	Introduction	133
	Materials and Methods	134

	Results	137
6.5	1987-1988 Field Studies	151
	Materials and Methods	151
	Results	154
6.6	Discussion	173
CHAPTER 7	**GENERAL CONCLUSIONS**	180
7.1	Over-View of Results	180
7.2	Conference Presentations	183
7.3	Research Papers to be published from this Study	185
BIBLIOGRAPHY		187
APPENDICES		216

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CHAPTER 1

Literature Review

1.1 Introduction

This literature review set out in this chapter covers three major areas related to the results of studies reported in this thesis, firstly the cultivation of subterranean clover (*Trifolium subterraneum*) and faba bean (*Vicia faba*), secondly information on viruses infecting these two legumes, especially those viruses recognised as causing damage in crop and forage legumes in Australia, and thirdly the dependent transmission of non-persistent viruses by aphids from mixed infections.

1.2 Cultivation of *Trifolium subterraneum* and *Vicia faba*

Subterranean clover (*Trifolium subterraneum*) is naturally distributed in the Mediterranean region and parts of Western Europe. In its native areas it grows as a weed or as a component of natural pastures on acid to neutral soils (Gladstones & Collins, 1983). It is also grown as a successful pasture species in Australasia, Israel, Portugal, Spain, USSR, South Africa and North America (Saxby, 1956 ; Morley, 1961 ; Knight *et al.*, 1982 ; Gladstones & Collins, 1983). Subterranean clover pastures have also been established in Argentina, Chile, Japan and at high altitudes in sub-tropical areas such as Kenya and Venezuela (Collins & Gladstones, 1984).

The success of subterranean clover as an annual pasture legume may be attributed to its productivity, persistence, ability to

increase soil fertility due to nitrogen fixation by association with *Rhizobium* spp., its tolerance of heavy grazing and its prevention of soil erosion. Furthermore, the dense root system is effective in building up the levels of nitrogen and organic matter in soil. It also tolerates waterlogging (Gladstones, 1975).

Subterranean clover was first recorded as a naturalized plant in Australia by Mueller in 1888 (Steele, 1930). It was probably introduced as a contaminant of agricultural seed imported from Europe, especially with crimson clover (*Trifolium incarnatum*) and red clover (*Trifolium pratense*) (Gladstones & Collins, 1983).

The potential of subterranean clover as a commercial pasture legume was first recognised by Howard, in 1906 (Hill, 1937). However, the areas sown dramatically increased after the 1930's when subterranean clover was demonstrated to be responsive to "top dressing" with superphosphate on infertile soils (Hudson, 1932 ; Donald & Williams, 1954). The subterranean clover pastures are predominantly located in wheat, sheep and fat lamb areas of southern Australia where the rainfall is less than 600mm annually and the soils are acidic and contained little organic matter (Wadham *et al.*, 1964).

Subterranean clover is now the most important pasture legume in temperate areas of southern Australia, particularly between latitudes 30° to 39° S (Morley, 1961 ; Powell, 1970 ; Gladstones & Collins, 1983 ; Cregan & Wolfe, 1988). It has been sown over more than 16 million hectares of dryland pasture (Cocks *et al.*, 1978). It is the basis of the improved pastures for the sheep and cattle industries and the nitrogen it fixes improves the productivity of the country's cereal industry (Gladstones & Collins, 1983).

Steele (1930) reported that the species was first noticed in

the southern region of Tasmania (Bothwell) in 1916. It was presumably a contaminant of other seeds imported from the mainland. Prior to 1928, the area sown to subterranean clover in Tasmania was less than 500 acres but an estimated area of 650,000 hectares had been established in Tasmania by 1986-1987 (Steele, 1930 ; Australian Bureau of Statistics, 1988). Subterranean clover grows on most soil types, from sandy soils to clay loams and is commonly sown either in permanent pastures or in rotations with cereal crops. The nitrogen fixed in the soil by the *Rhizobium* associated with it can be used by subsequent crops and the plant itself provides nutritious feed for livestock (Hudson, 1932 ; Cocks *et al.*, 1978).

Vicia faba is widely grown in temperate areas as well as in the tropical, subtropical and arid areas of the world as a cool season crop. There are many common names for this species but most of them refer to a particular subgroup rather than to the species. The smaller-seeded types, vars. *minor* and *equina* are often called field beans in the United Kingdom and Europe while the large-seeded var. *major* types are usually known as broad beans (Hawtin & Hebblethwaite, 1983). Other common names include Egyptian, horse, longpod, Spanish, tick, Vicia and Windsor beans. The term "faba bean" has now been widely adopted to indicate all the different types (Bond *et al.*, 1985).

In earlier classifications seed size was one of the main criteria for division of the species into a range of types but this is now no longer accepted because there are some small seeded broad beans and large seeded horse beans (Lawes *et al.*, 1983). The main classification of cultivars is now based on their use, hence the broad beans grown as a vegetable are distinguished from the field beans grown for animal feed which in turn are further subdivided

into tick beans and horse beans. Broad beans are used as a fresh vegetable in temperate areas and also for canning and freezing. In some arid and tropical regions the dried mature seeds are stored for use when fresh vegetables are in short supply (George, 1985).

The original region of *V. faba* remains unknown. Linnaeus (1738) and Abdalla (1979) indicated that the faba bean originated in Egypt with other possibilities being western Asia (Cubero, 1974) or central Asia (cited by Hawtin & Hebblethwaite, 1983 ; Bond *et al.*, 1985).

V. faba is now one of the most important grain legume crops in the world being grown on 3.6 million hectares in about 50 countries by 1981. The *minor* and *equina* types are most prevalent in northern Europe, the Nile valley, Ethiopia, Afghanistan and North America. In most other regions, such as western Asia, China, the Mediterranean Basin and South America, var. *major* is more common (Bond *et al.*, 1985).

Owing to the need to diversify agricultural production, the faba bean has recently been established in Australia as a commercial crop although it has been cultivated for many years as a home garden vegetable (Hawtin & Hebblethwaite, 1983). The crop is sown mainly in winter, on less than 10,000 hectares annually, in the southern temperate region of the country (Laurence, 1979 ; Baldwin, 1980 ; Mahoney, 1987).

In Tasmania, faba beans are grown mainly in the north-western region of Tasmania. *V. faba* var. *major* (broad bean) is harvested green for consumption as a vegetable from a total area of about 400 hectares and almost all of that produce is processed by canning or freezing with a total annual production of 1300 tonnes (3.3 tonnes/hectare). A small proportion of the harvest is sold as a

fresh vegetable (Australian Bureau of Statistics, 1988). *V. faba* var. *minor* (tick bean) is grown to a minor extent, as green manure crops in orchards and as food for horses (McCutcheon, 1989).

Viruses affecting subterranean clover and faba bean have been reported from many different parts of the world. In the early 1920's, "mosaic disease" on faba bean crops was recorded in the United States (Dickson, 1921 ; Elliot, 1921), Japan (Fukushi, 1930), the Netherlands, Germany and Bermuda (Bos, 1982). Similarly, infection of subterranean clover with mosaic disease was reported in Australia more than forty years ago (Aitken & Grieve, 1943 ; Norris, 1943). Since those early reports of mosaic diseases, a total of ten viral diseases have now been recorded in subterranean clover pastures with approximately 40 such diseases recorded in faba bean crops (Cockbain, 1983 ; Edwardson & Christie, 1986a, 1986b ; Johnstone & McLean, 1987) (Table 1.1).

A variety of techniques are used to identify these viruses. A few of the viruses infecting subterranean clover and faba beans can be diagnosed merely by the symptoms they cause, but most can only be identified by further testing to determine, for example, the morphology of their capsids, their modes of transmission, host ranges and their serological properties (Bos, 1982). Many viruses can now be identified rapidly using modern serological techniques such as enzyme-linked immunosorbent assay and immune electron microscopy, or by nucleic acid hybridization analysis (Bos, 1982 ; Cockbain, 1983).

1.3 Characterisation of viruses infecting *Trifolium subterraneum* and *Vicia faba*.

Although many viruses naturally infect both *T. subterraneum*

TABLE 1.1

Viruses reported from naturally infected *Trifolium subterraneum* L. and *Vicia faba* L..

Name of virus	Virus Group	Species Infected	Distribution	Reference
Alfalfa mosaic virus	Alfalfa	*T. subterraneum*	world wide	Kreitlow & Price, 1949
		V. faba		Cockbain, 1983
Bean common mosaic virus	Potyvirus	*V. faba*	Syria, U.S.A.	Cockbain, 1983
Bean leafroll virus (synonym : pea leafroll virus)	Luteovirus	*V. faba*	Europe, Iran, USA	Bos, 1982
Bean yellow mosaic virus (synonyms : pea mosaic strain, *Phaseolus* virus 2)	Potyvirus	*T. subterraneum*	Australia, USA	Norris, 1943
		V. faba	world wide	Cockbain, 1983
Bean yellow veinbanding	unknown, spherical	*V. faba*	England	Cockbain, 1978
Beet western yellows virus	Luteovirus	*T. subterraneum*	Australia, USA New Zealand	Johnstone & McLean, 1987
		V. faba	Europe, USA	Bos, 1982
Broad bean mild mosaic virus	Potexvirus	*V. faba*	China	Cockbain, 1983
Broad bean mosaic virus	unknown, spherical	*V. faba*	Algeria, India	Cockbain, 1983

Broad bean mottle virus	Bromovirus	*T. subterraneum* *V. faba*	Africa, Europe Africa, UK, India	Gibbs, 1972 Bos, 1982
Broad bean necrosis virus	Tobravirus or Tobamovirus	*V. faba*	Japan	Inouye & Nakasone, 1980
Broad bean severe chlorosis virus	Potyvirus	*V. faba*	USA	Thottappilly *et al.*, 1975
Broad bean stain virus	Comovirus	*V. faba*	Australia, Europe, Morocco	Bos, 1982
Broad bean true mosaic virus	Comovirus	*V. faba*	Australia, USSR, Europe, Egypt	Cockbain, 1983
Broad bean wilt virus	Fabavirus	*V. faba*	Africa, Asia, Australia, Europe, North America	Büchen-Osmond *et al.*, 1988
Broad bean yellow vein virus	Rhabdovirus	*V. faba*	Japan	Natsuaki, 1981
Clover yellow vein virus	Potyvirus	*T. subterraneum* *V. faba*	Australia Netherlands, Tasmania	Johnstone & McLean, 1987 Cockbain, 1983
Cowpea mosaic virus	Comovirus	*V. faba*	West Indies	Cockbain, 1983
Cucumber mosaic virus	Cucumovirus	*T. subterraneum* *V. faba*	Australia Europe, Iran, Morocco	Jones, 1988 Bos, 1982
Legume yellows virus	Luteovirus	*V. faba*	USA	Duffus, 1979

Milk-vetch dwarf virus	Luteovirus	*V. faba*	Japan	Ohki *et al.*,1975
Pea early-browning virus	Tobravirus	*V. faba*	England, Morocco Poland	Cockbain, 1983
Pea enation mosaic virus	ungrouped, spherical	*T. subterraneum* *V. faba*	USA China, Europe, USSR, USA	McWhorter & Cook, 1958 Cockbain, 1983
Peanut mottle virus	Potyvirus	*T. subterraneum*	USA	Khan & Demski, 1981
Pea pimple pod virus	Phytoreovirus	*V. faba*	Tasmania	Wade, 1951
Pea seed-borne mosaic virus	Potyvirus	*V. faba*	Europe, Japan	Cockbain, 1983
Red clover vein mosaic virus	Carlavirus	*V. faba*	East Germany, USA	Bos, 1982
Soybean dwarf virus (synonym : subterranean clover red leaf virus)	Luteovirus	*V. faba* *T. subterraneum*	Japan, Australia, New Zealand Australia, New Zealand	Tamada & Kojima, 1977 ; Bos, 1982 Kellock, 1971 Wilson & Close, 1973
Subterranean clover mottle virus	Sobemovirus	*T. subterraneum*	Australia	Graddon *et al.*, 1984
Subterranean clover stunt virus	ungrouped, spherical	*T. subterraneum* *V. faba*	Australia Australia	Grylls & Butler, 1956 Smith, 1966

Tomato spotted wilt virus	ungrouped, spherical	*V. faba*	Australia, China, India, South Africa	Cockbain, 1983
Vicia cryptic virus	ungrouped, spherical	*V. faba*	Scotland	Kenten *et al.*, 1978
Watermelon mosaic virus	Potyvirus	*V. faba*	Japan	Cockbain, 1983
White clover mosaic virus	Potexvirus	*T. subterraneum*	Australia, New Zealand	Johnstone & McLean, 1987
		V. faba	East Germany, Japan New Zealand	Bos, 1982

and *V. faba* (Table 1.1), few viruses have been found in these two legume crops in Australia. Those causing disease problems in Australia include cucumber mosaic virus, potyviruses (bean yellow mosaic virus and clover yellow vein virus), soybean dwarf virus (syn. subterranean clover red leaf virus) and subterranean clover stunt virus.

Cucumber mosaic virus.

Introduction

Cucumber mosaic virus (CMV) was independently described by Doolittle and Jagger as the cause of a disease in cucumber (*Cucumis sativus*) in the U.S.A. in 1916 (Kaper & Waterworth, 1981). Many synonyms have been listed, including cucumber virus 1, *Cucumis* virus 1, *Marmor cucumis* and tomato fern leaf virus (Francki *et al.*, 1979).

CMV was probably introduced to Australia with seed imports during the last two centuries. It is now widespread in most states and is especially important in South Australia and Western Australia where the virus seriously damages lupins (*Lupinus* spp.) and lentils (*Lens culinaris*) (Büchen-Osmond *et al.*, 1988). CMV was first reported infecting subterranean clover cv. Enfield in 1982 in Western Australia (Johnstone & McLean, 1987). Subsequently, CMV infection was found severely damaging several other cultivars including Esperance, Nangeela and Northam. In Victoria CMV was found on subterranean clover, on cv. Enfield at Bendigo, Maffra, Melbourne, Tatura and Werribee. Other cultivars susceptible to CMV included Woogenellup and Yarloop (Johnstone & McLean, 1987). In South Australia CMV was isolated from *V. faba* var. major (Büchen-Osmond *et al.*, 1988). The virus caused a slight stunting and nondescript yellowing on white clover (*Trifolium repens*), a

perennial species which is probably important as a primary source of the virus for spread to subterranean clover (Garrett, 1985).

Host range and symptoms

CMV has a wide host range especially among species adapted to temperate regions of the world. It infects cereals, forage crops, woody and herbaceous ornamentals as well as vegetable and fruit crops. Some 470 plant species representing 67 families are reported to be natural hosts of CMV, including 85 species of the Fabaceae (Kaper & Waterworth, 1981 ; Edwardson & Christie, 1986a). The most common symptom incited by CMV is mosaic but the severity of the diseases induced may range from no obvious symptoms in some crops to death of infected plants in others (Gracia & Feldman, 1977 ; Kaper & Waterworth, 1981). Other symptoms on the crops include blight, fern leaf, flower breaking and ring-spot (Fulton, 1950 ; Smith, 1972).

CMV causes stunting, leaflet downcurling, mottle or mosaic on subterranean clover, whereas symptoms on faba beans are brown or reddish-brown local lesions, sometimes accompanied by systemic necrosis and stunting (Cockbain, 1983 ; Garrett, 1985).

Diagnostic species (Francki *et al.*, 1979 ; Büchen-Osmond *et al.*, 1988)

Chenopodium amaranticolor, C. quinoa. Chlorotic or necrotic local lesions.

Cucumis sativus. Systemic mosaic and stunting.

Lycopersicon esculentum. Mosaic, fern leaf symptoms.

Nicotiana glutinosa. Variable symptoms from mild to severe mosaic depending on strain.

Propagation species (Francki *et al.*, 1979)

Nicotiana spp. are suitable for maintaining virus isolates, particularly *N. clevelandii, N. glutinosa* and *N. tabacum*. *Cucurbita pepo* is also useful as a propagation host.

Transmission

CMV is transmitted naturally via seeds in some species and by more than 60 aphid species in a non-persistent manner (Kennedy *et al.*, 1962). Moreover, all instars can acquire the virus from infected plants and inoculate it into healthy plants in less than one minute of feeding (Francki *et al.*, 1979). The aphids retain the virus for less than four hours, and it is not transmitted to progeny aphids (Simon, 1955 ; Pirone & Harris, 1977). Efficiency of transmission varies with the aphid species and host plants (Simon, 1957). For example, *Aphis gossypii* and *Myzus persicae* are regular vectors of CMV in many crops (Kennedy *et al.*, 1962) whereas the foxglove aphid (*Aulacorthum solani*), the blue green lucerne aphid (*Acyrthosiphon kondoi*) and the pea aphid (*Acyrthosiphon pisum*) are probably the most important vectors of this virus in pasture legumes in Australia (Johnstone & Barbetti, 1987). The importance of CMV in legumes in Australasia has increased since the arrival of the lucerne and pea aphids between 1977 and 1981 (Ashby, 1980).

CMV is transmitted through the seed of 19 species (Neergaard, 1977). The seed transmission rate varies depending on the host e.g. 2% in tomatoes, 7% in pumpkins, 6.4% in lupins and 0.3 to 30% amongst bean varieties (Edwardson & Christie, 1986a). Transmission through seeds of subterranean clover cultivars were 1% in cv. Clare, Esperance and Meteora, 2 - 8% in cv. Enfield, 4% in cv. Mt. Barker; and 5% in cv. Nangeela (Jones, 1988). No seed transmission

has been reported in *V. faba*. CMV is also transmitted through the seed of several weed species, including *Cerastium* sp. (2%), *Stellaria* sp. (21 to 40%) and *Echinocystis* sp. (9 to 95%) (Neergaard, 1977).

Properties of Particles

CMV, a member of cucumovirus group, has isometric particles about 28 to 30nm in diameter (Francki *et al.*, 1979 ; Kaper & Waterworth, 1981 ; Büchen-Osmond *et al.*, 1988). It contains single stranded RNA and the particles sediment uniformly at about 100s (Francki *et al.*, 1966). There are four major RNA components in the particles (RNA-1 to RNA-4) having molecular weights of about 1.35, 1.03, 0.75 and 0.35 x 10^{-6} respectively, but these vary somewhat depending upon the virus strain (Francki *et al.*, 1979 ; Symons, 1985). RNA-1, 2 and 3 are the three largest RNAs and carry all the information needed for virus infection and replication while RNA-4 is a subgenomic fragment of RNA-3. The gene for viral coat protein is carried on RNA-3 and RNA-4 (Gould *et al.*, 1978 ; Schwinghamer & Symons, 1977).

RNA-5, a smaller minor RNA fragment, with molecular weight about 0.11 x 10^{-6}, has been detected in some isolates of CMV (Kaper & Waterworth, 1977). Gould *et al.* (1978) showed that RNA-5 behaved as a satellite RNA associated with CMV. Two distinct types of RNA-5 were distinguished. The first consisted of specific cleavage products of RNAs 1-4. The second was designated as true satellite RNA because it had no nucleotide sequence homology in common with RNAs 1-4 and depended on CMV for its multiplication (Gould *et al.*, 1978). The satellite RNA's associated with different CMV isolates have variations in their base sequences that lead to either the

enhancement or attenuation of the disease symptoms induced by CMV. For example, a CMV isolate containing the satellite RNA CARNA 5 was observed to induce a lethal necrotic disease in tomato instead of the chlorosis and fern leaf symptoms commonly induced by CMV alone (Kaper & Waterworth, 1981).

Serological relationships

The immunogenicity of CMV is poor but it can be enhanced by fixation with 0.2% formaldehyde (Francki & Habili, 1972). Enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM) are now widely used for detecting the virus because these systems are very sensitive (Kaper & Waterworth, 1981). Furthermore, CMV has also been detected *in situ* with the fluorescent antibody procedure (Otsuki & Takebe, 1973).

CMV is related to the other members of the cucumovirus group including peanut stunt virus and tomato aspermy virus. The degree of relationship reported varies according to the virus isolates that are compared and the experimental conditions adopted for undertaking the tests (Kaper & Waterworth, 1981).

Potyviruses

Introduction

Many potyviruses are able to infect *T. subterraneum* or *V. faba* but only bean yellow mosaic virus (BYMV), clover yellow vein virus (CYVV) and pea mosaic virus (PMV, a strain of BYMV) commonly infect these two legumes naturally in temperate regions (Table 1.1).

BYMV was first found in *Phaseolus vulgaris* and described by Pierce (1934). Synonyms of BYMV include bean virus 2, *Phaseolus* virus 2 and gladiolus mosaic virus (Bos, 1970). It has a worldwide

distribution, infects many legume crops and generally causes mosaic symptoms (Cockbain, 1983). The virus has been recorded in subterranean clover in Australia and U.S.A. (Norris, 1943).

The virus was probably introduced into Australia by seed or in plant material such as corms of gladiolus during the last two centuries (Büchen-Osmond *et al.*, 1988). The first record of a mosaic disease of *T. subterraneum* in the field was from Western Australia in 1939. It was identified as PMV on the basis of its effects on lupins (*Lupinus* spp.) and pea (*Pisum sativum*) (Norris, 1943). Since then the virus has been recorded infecting the clovers in Victoria (Aitken & Grieve, 1943), Tasmania (White, 1945), South Australia (Watson, 1949) and the Australian Capital Territory and New South Wales (Hutton & Peak, 1954). Natural infection of *V. faba* by BYMV in Australia has been recorded in Queensland, South Australia, Tasmania and Western Australia (Büchen-Osmond *et al.*,1988).

CYVV was first described by Hollings and Nariani (1965) from white clover. It has been reported in *Trifolium* species in Australia, Europe, New Zealand and North America (Hollings & Nariani, 1965 ; Pratt, 1969 ; Alconero, 1983 ; Forster & Musgrave, 1985). Natural infection of *T. subterraneum* and *V. faba* with CYVV was only reported from Australia and the Netherlands (Table 1.1). In Australia it was found in subterranean clover from Victoria and in *V. faba* from Tasmania, Victoria and Western Australia (Büchen-Osmond *et al.*, 1988).

Host ranges and symptoms

BYMV is distributed throughout the world and infects at least 230 species in 74 genera of 20 families, including 157 species in 30

genera of the Fabaceae (Edwardson & Christie, 1986b). It causes mosaic diseases in most legume hosts, with the severity of the symptoms dependent on the strain of the virus. Other naturally infected species commonly found include *Gladiolus* sp., *Freesia* sp., *Lupinus luteus, Robinia pseudo-acacia, Trifolium pratense, Trigonella foenum-graecum* and *Vicia sativa* with necrotic lesions or streaks, mosaic, mottle, and distortion of the leaves and stems (Bos, 1970 ; Büchen-Osmond *et al.*, 1988 ; Jones & Diachun, 1976).

Diagnostic host species for BYMV

Chenopodium amaranticolor chlorotic or necrotic lesions, occassional systemic vein yellowing or vein banding and leaf malformation.

Chenopodium quinoa necrotic or chlorotic local lesions; not systemic.

Gomphrena globosa local infection, often with necrotic local lesions.

Phaseolus vulgaris necrotic or chlorotic local lesions, systemic mosaic, leaf curling and malformation: symptoms depend on host variety and virus strain.

Pisum sativum "Perfection" type peas are usually immune but others show symptoms of vein chlorosis followed by mosaic.

Vicia faba green or yellow mosaic.

Maintenance and propagation host species for BYMV

Nicotiana clevelandii, Phaseolus vulgaris, Pisum sativum and *Vicia faba* are good sources for virus purification, and also for maintaining virus cultures (Bos, 1970 ; Büchen-Osmond *et al.*, 1988).

CYVV has a narrower host range. It is known to infect 48 species in 21 genera of 8 families, including 30 species in 9 genera of the Fabaceae (Edwardson & Christie, 1986b). In the field it is mainly confined to *Trifolium* species, especially *T. repens* (Barnett *et al.*, 1983). It causes mild veinal yellowing and mottling in *Trifolium* spp., especially in white clover. It also infects *Coriandrum sativum*, *Daucus carota*, *Lupinus* sp. and *Glycine max* in nature (Büchen-Osmond *et al.*, 1988). CYVV generally causes mosaic and vein clearing in subterranean clover but some isolates from Victoria, Australia, caused a lethal necrosis (Johnstone & McLean, 1987). Symptoms on *V. faba* infected with CYVV depend upon the plant variety and virus strain. Most commonly it induces necrotic local lesions on the inoculated leaves followed by systemic mosaic, leaf and stem necrosis, wilt and premature death (Cockbain, 1983).

Diagnostic host species for CYVV

Chenopodium amaranticolor necrotic local lesions followed by abscission of the inoculated leaves; no systemic infection.

Chenopodium quinoa fawn, necrotic local lesions, systemic chlorosis, fleck and necrotic spots develop in 2 weeks.

Nicotiana clevelandii chlorotic or necrotic local lesions, systemic mosaic followed by necrosis.

Nicotiana tabacum (cv. White Burley) chlorotic local lesions; no systemic infection.

Trifolium repens mosaic and chlorotic bands or asymptomatic.

Maintenance and propagation host species for CYVV

Nicotiana clevelandii is a good source of virus for purification, and for maintaining virus cultures (Hollings & Stone, 1974 ;

Büchen-Osmond *et al.*, 1988).

CYVV can be distinguished from BYMV/PMV on the basis of biological properties, as most isolates infect white clover whereas BYMV/PMV isolates do not (Hagedorn & Walker, 1950 ; Pratt, 1969 ; Barnett & Gibson, 1975).

Transmission

BYMV/PMV and CYVV are transmissible by mechanical inoculation and by aphid vectors in a non-persistent manner. More than 40 aphid species can transmit BYMV, including *Acyrthosiphon pisum*, *Aphis craccivora*, *Aphis fabae*, *Macrosiphum euphorbiae* and *Myzus persicae* (Bos, 1970 ; Edwardson & Christie, 1986b). The aphid species known as vectors of CYVV are *A. craccivora*, *A. pisum*, *A. solani* *M. euphorbiae* and *M. persicae* but not *A. fabae* (Hollings & Nariani, 1965 ; Singh & Lopez-Abella, 1971).

BYMV is seed-borne in lupins, Bokhara clover (*Melilotus albus*), red clover, French bean and faba bean (Neergaard, 1979 ; Edwardson & Christie, 1986b), but does not seem to be transmitted through the seed of subterranean clover (Norris, 1943). It is occasionally seed-borne in *V. faba* but the rate of transmission is usually very low (Kaiser, 1972). CYVV is not transmitted through seed of *P. vulgaris* cv. Prince. There are no reports on seed transmission of CYVV in subterranean clover and faba bean.

Properties of particles

BYMV/PMV and CYVV have flexuous filamentous particles about 700-850nm long and 12-15nm wide (Bos, 1970 ; Hollings & Stone, 1974). They are known to have a helical symmetry with a pitch of

approximately 3.4*nm*. The particles contain 5% nucleic acid, 95% protein and they sediment as a single component (140-157s for BYMV and 159 for CYVV). The A_{260}/A_{280} ratio of unfractionated preparations is 1.08 (BYMV) and 1.12 (CYVV) (Büchen-Osmond *et al.*, 1988). As with some other potyviruses, the length and flexuousness of the particles in plant extracts is affected by the concentration of magnesium ions in the extracting fluid. The magnesium ions apparently form bridges between carboxyl groups of capsid protein and modify the conformation of the sub-units, causing the particles to increase in the length and become more rigid (Govier & Woods, 1971).

Serological relationships

The virus preparations are strongly immunogenic. Serological tests with these viruses in gels require that they be sonicated (Tomlinson & Walkey, 1967) or treated with 0.5% (w/v) SDS so that they are broken down and will diffuse (de Bokx & Huttinga, 1981). Micro-precipitin and precipitin tube tests are also satisfactory. In addition, these potyviruses can be detected by immune electron microscopy, enzyme-linked immunosorbent assays and latex agglutination (Büchen-Osmond *et al.*, 1988).

Isolates of BYMV, CYVV and PMV have been distinguished on the basis of their host ranges, the symptoms they cause (Bos *et al.*, 1974 ; Jones & Diachun, 1977 ; Hampton *et al.*, 1978) and the serological specificities of their particles (Moghal & Francki, 1976a). However variation has been reported in the biological properties of BYMV isolates (Musil, 1975). Moghal and Francki (1976a, 1981) reported that it was difficult to distinguish BYMV and PMV isolates by intra-gel immunodiffusion absorption tests, coat

protein analyses, particle length measurements or by the cytology of infected host plants. In recent studies, the amino acid composition of the coat proteins (Randles *et al.*, 1980) and molecular hybridization analyses (Abu-Samah & Randles, 1981 & 1983 ; Barnett *et al.*, 1987) were found to be potentially more useful to differentiate BYMV isolates.

Yield effects and control

a) *Trifolium subterraneum*: Potyvirus infections of clover in Australia cause sporadic but important losses. All the early commercial cultivars were infected by BYMV (Aitken & Grieve, 1943) although their symptoms were different. Hutton and Peak (1954) reported that growth reductions of five cultivars varied between 26% and 77%. Infected plants matured earlier than the normal plants and often failed to set seed. Mixed infections of BYMV and SCSV in some cultivars in New South Wales almost totally stopped seed development (Grylls & Peak, 1969). These workers found five seedlines that were highly resistant to infection with these two viruses. The spread of BYMV in the field was related to the aphid flight activity, particularly in the spring. There are many aphid species known as vectors of BYMV, including *A. pisum*, *A. craccivora*, *A. gossypii*, *A. solani* and *M. euphorbiae* (Jayasena & Randles, 1984).

White clover is probably the prime source of infection of CYVV for spread to subterranean clover. Foxglove aphids commonly infest white clover and they may be the major vector in the field. Pea aphids probably have a minor role because of their lower population density on the white clover (Ashby, 1980).

b) *Vicia faba*: The effect of BYMV depends on the time of infection and on the virus strain. Broad bean plants inoculated

with BYMV before, during or after flowering showed decreased yields of 44%, 42% and 23% respectively (Kaiser, 1973). Broad bean plants in Sudan which were naturally infected with PMV yielded 67-82% fewer pods than healthy plants (Cockbain, 1983). Additionally, BYMV reduced yield solely by decreasing the numbers of pods per plant and numbers of seeds per pod in glasshouse and in field experiments (Bailiss & Senananyake, 1984). Conversely, PMV had little effect on the yield of broad beans in New Zealand (Chamberlain, 1936).

No varieties of *V. faba* were found resistant to BYMV in Israel, Egypt, Iran and Poland. However in the Netherlands the cultivar Beryl seemed resistant to infection and the cultivars Felissa, Felix Suprifin were moderately resistant (Cockbain, 1983).

Although insecticides are not usually effective in controlling the spread of non-persistent aphid-borne viruses, some treatments have been found to decrease or delay the spread of BYMV. In trials in East Germany aerial spraying of field beans with dimethoate about 20 days before flowering followed by endosulfan 11-46 days later reduced the mean incidence of BYMV from 16 to 6% in 1974, from 20 to 3% in 1976 and from 41 to 17% in 1977 (Cockbain, 1983).

Other methods of attempting to control BYMV include manipulation of sowing date (Abu Salih *et al.*, 1973), spraying with mineral oils, mulching with aluminium polythene film, ensuring that crops are not grown near overwintering sources of the viruses and keeping seed crops as virus-free as possible (Cockbain, 1983).

Soybean dwarf virus (syn. subterranean clover red leaf virus)

Introduction

Subterranean clover red leaf virus (SCRLV) was first found in *T. subterraneum* in Victoria, Australia, in 1965 (Anon., 1968).

Apart from Victoria, the virus is known to occur only in the other southern Australian states and in New Zealand (Wilson & Close, 1973 ; Johnstone & Barbetti, 1987). *V. faba* plants infected with SCRLV have been identified in the field in South Australia, Tasmania and Victoria (Büchen-Osmond *et al.*, 1988).

Information on the biological properties of Australasian SCRLV isolates was reported by Kellock (1971) and Wilson and Close (1973). Subsequently, the morphology of SCRLV was observed in thin sections of infected plants (Jayasena *et al.*, 1981) and the virus was purified and characterised as a member of luteovirus group (Ashby & Kyriakou, 1982; Johnstone *et al.*, 1982). A disease of soybean [soybean dwarf virus (SDV)] reported in Japan (Tamada *et al.*, 1969; Tamada, 1970) appeared identical to SCRLV when compared in serological tests (Ashby & Kyriakou, 1982). The host ranges and vector specificities of these two viruses were also similar (Tamada, 1970; Johnstone *et al.*, 1984a ; Ashby & Johnstone, 1985). It now appears that SCRLV is probably a strain of SDV (Waterhouse *et al.*, 1988). This review therefore covers information on SCRLV and SDV under the SDV acronym.

SDV has been reported to infect many legume species including broad bean and subterranean clover in Australia, Japan, New Zealand and U.S.A. (Büchen-Osmond *et al.*, 1988).

Host range and symptoms

SDV infects at least twenty-three species of papilionoid legumes (Tamada, 1970; Kellock, 1971; Johnstone *et al.*, 1984a). A few non-legumes are also infected including *Beta vulgaris*, *Erodium cicutarium*, *E. moschatum*, *Gomphrena globosa*, *Lactuca sativa*, *Malva parviflora*, *Rumex obtusifolius*, *Tetragonia expansa* and *Zinnia*

elegans (Ashby *et al.*, 1979; Johnstone *et al.*, 1984a). The symptoms of virus infection are generally stunting, chlorosis and interveinal yellowing or marginal reddening of older leaves (Tamada & Kojima, 1977). Different strains of SDV in Japan are characterised on the basis of their host ranges and disease syndromes (Tamada, 1973). Isolates of SDV from Australia and New Zealand also differed slightly in the host ranges, particularly with respect to infection of lucerne (*Medicago sativa*) and docks (*Rumex* spp.) (Johnstone *et al.*, 1984a).

Diagnostic host species (Tamada & Kojima, 1977; Büchen-Osmond *et al.*, 1988).

Beta vulgaris systemic yellowing.

Erodium cicutarium systemic reddening.

Glycine max interveinal yellowing, leaves rolled down, stunting with shortened petioles and internodes.

Lupinus cosentinii systemic yellowing and reddening.

Phaseolus vulgaris (cvs. Galatin 50, Tendergreen, Topcrop) interveinal chlorosis, leaf puckering, leaves rolled down.

Pisum sativum (cvs. Laxton's Superb, Onyx, Puget) general yellowing, leaves brittle, rolling down.

Trifolium incarnatum stunting, chlorosis and marginal reddening of older leaves.

Trifolium subterraneum leaf reddening developing from the margins of the leaflets.

Vicia faba interveinal chlorosis, leaves rolled upwards and with a harsh feel.

Maintenance and Propagation host species (Tamada & Kojima, 1977; Büchen-Osmond *et al.*, 1988).

Glycine max (cv. Shiro Tsurunoko) for SDV isolates in Japan.

Pisum sativum (cvs. Onyx, Puget) for SDV isolates in Australia, New Zealand and U.S.A.

Red clover (*Trifolium pratense*) and white clover (*T. repens*) do not commonly display symptoms when infected with SDV. These two species, perennial pasture legumes, serve as major sources of virus infection in various regions from which spread occurs to annual legumes in spring, including subterraneum clover and faba bean (Johnstone & McLean, 1987).

Transmission

SDV is not transmitted by mechanical inoculation or by seeds but it is transmissible in a persistent manner by specific aphid vectors (circulative, non-propagative). Acquisition and inoculation access periods for *A. solani*, the former principal vector of SDV, were both 48 to 72 hours (Büchen-Osmond *et al.*, 1988). The minimum acquisition access period is between 30 to 60 minutes (Tamada, 1970) or 6 hours (Kellock, 1971) and minimum inoculation access period is between 10 and 30 minutes (Tamada, 1970 ; Kellock, 1971). The minimum latent period in the vector is between 15 and 27 hours, depending on the length of the acquisition access period. Young nymphs transmit the virus more efficiently than later instars and alates. In serial transmission tests, *A. solani* retained the ability to transmit the virus after moulting for periods up to 40 days (Kellock, 1971 ; Tamada & Kojima, 1977). *A. solani* was the most important vector in Australia, New Zealand and Japan. Some

other aphid species are also reported to be vectors in transmission of SDV (Table 1.2). The lily aphid (*Aulacorthum circumflexum*) is also an efficient vector of Australian isolates but it is unlikely to be important in nature (Helms *et al.*., 1983). Some SDV-like isolates recovered from legumes in California were transmitted specifically by the pea aphid (*A. pisum*) (Johnstone *et al.*, 1984b).

Properties of particles

SDV is a luteovirus with isometric particles about 25 to 28 nm in diameter. The particles consist of 28 to 30% nucleic acid and 70 to 72% protein. The A_{260}/A_{280} ratio of purified preparations is about 1.85 to 1.96, depending upon the virus isolate (Tamada & Kojima, 1977; Büchen-Osmond *et al.*, 1988).

Serological and Relationships

The virus is strongly immunogenic. It can be detected by agar gel diffusion testing, immune electron microscopy, enzyme-linked immunosorbent assay and immune density gradient centrifugation (Büchen-Osmond *et al.*, 1988).

There are many reports on the serological relationships between SDV and other luteoviruses. As previously mentioned SDV is serologically very closely related to SCRLV and it was also found to be related to some extent to beet western yellows, barley yellow dwarf (MAV and RPV isolates), tobacco necrotic dwarf and turnip yellows viruses (Rochow & Duffus, 1981; Waterhouse *et al.*, 1988). SCRLV is also serologically related to beet western yellows, bean leaf roll, legume yellows, potato leaf roll and tomato yellow top viruses (Waterhouse *et al.*, 1988). However, some Tasmanian isolates of SCRLV were quite distinct from isolates of beet western yellows

TABLE 1.2

Aphid vectors of different isolates of SDV.

Virus isolate (Source)	Prime vector	Other vectors	Non-vectors	References
SCRLV-NZ (New Zealand)	*Aulacorthum solani*	*Acyrthosiphon pisum* *Macrosiphum euphorbiae*	*Aphis craccivora* *Myzus persicae*	Wilson & Close, 1973 ; Ashby, 1980
SCRLV-T (Australia)	*A. solani*	*Aulacorthum circumflexum*	*Acyrthosiphon kondoi* *A. pisum* *Aphis craccivora* *Dysaphis aucupariae* *Lipaphis erysimi* *M. euphorbiae* *M. persicae*	Johnstone & Patten, 1981 ; Helms *et al.*, 1983
SDV-J (Japan)	*A. solani*		*A. craccivora* *A. glycines* *A. kondoi* *A. pisum* *M. euphorbiae* *M. persicae*	Tamada & Kojima, 1977
AP-45 (USA)	*A. pisum*		*A. craccivora* *A. solani*	Johnstone *et al.*, 1984b

virus from Tasmania and U.S.A., legume yellows virus (U.S.A.) and potato leaf roll virus (Tasmania) when compared by DAS-ELISA (Johnstone & Duffus, 1984).

Occurrence and control

White clover seems to be the most important reservoir of SDV in Australia and Japan. Surveys of white clover in Japanese, New Zealand and Tasmanian pastures indicated levels of infection of 30%, 60% and 37% respectively (Tamada, 1975 ; Ashby, 1980 ; Johnstone & Duffus, 1984). Furthermore, A. *solani*, the most important vector, prefers white clover as an overwintering and oversummering host (Johnstone, 1978 ; Ashby *et al.*, 1979). In New Zealand, more than 30% of individual A. *solani* trapped were able to transmit SDV to subterranean clover (Wilson & Close, 1973). Under Tasmanian conditions, most of A. *solani* caught in broad bean crops seemed to be carrying SDV (Johnstone & Rapley, 1981).

The relationship between the time of seedling emergence and peaks of aphid flight activity influence the spread of SDV into broad bean crops (Johnstone & Rapley, 1979, 1981 ; Jayasena & Randles, 1984). The relative importance of spread by alatae and apterae into and within crops depended upon the time of year the crops were sown (Johnstone & Rapley, 1979).

Methods of attempting to control the spread of SDV are manipulation of sowing date (Johnstone & Rapley, 1979), application of aphicides such as demeton-S-methyl (Johnstone & Rapley, 1981) and the synthetic pyrethroid deltamethrin (Johnstone, 1984) and by selecting resistant varieties (Cockbain,1983).

Subterranean clover stunt virus

Introduction

Subterranean clover stunt virus (SCSV) was first found in subterranean clover (*T. subterraneum*) in southern States of Australia about 1950 (Grylls & Butler, 1956 ; Harvey, 1958 ; Anon., 1962). This virus has not been found outside Australia (Johnstone & McLean, 1987). It has only been recorded infecting *V. faba* in New South Wales (Grylls, 1972) and Tasmania (Johnstone, 1978).

Host range and symptoms

SCSV is known to infect 42 species in 16 genera of legumes (Edwardson & Christie, 1986a) including *T. repens*, *T. subterraneum*, *P. vulgaris*, *P. sativum* and *V. faba* (Smith, 1966). Symptoms caused by SCSV are commonly stunting, systemic reddening or yellowing, leaf rolling and leaf distortion (Boswell & Gibbs, 1983).

Symptoms of the disease on subterranean clover include severe stunting of the plants, leaf distortion, cupping and yellowing of new growth and reddening of older leaves (Grylls & Butler, 1959). The effects of SCSV on most cultivars of *T. subterraneum* are very severe. O'Loughlin (1958) and Grylls and Peak (1960) reported that cv. Tallarook was either very resistant or immune to SCSV (isolates from Canberra and Victoria). However this cultivar was susceptible to some Tasmanian isolates of the virus (Johnstone, 1983b).

Infected *V. faba* plants display leaf chlorosis, rolling, shortening of petioles and internodes, and leaves reduced in size (Smith, 1966; Grylls, 1972).

Diagnostic host species

Astragalus sinicus stunting, leaf yellowing, size reduction and

rolling down.

Medicago hispida severe stunting, rosette appearance, puckering and marginal chlorosis and reddening of older leaves.

Phaseolus lathyroides severe stunting and marked reduction of leaf size following marginal chlorosis and puckering of terminal leaves.

Trifolium subterraneum severe stunting, leaf distortion, cupping and yellowing of new growth and redding of older leaves as described previously.

Maintenance and propagation host species

Pisum sativum (cv. Greenfeast) for propagation only.

T. subterraneum (cv. Mt. Barker) for maintenance and propagation.

(Reference: Büchen-Osmond *et al.*, 1988)

Transmission

SCSV is not transmissible by mechanical inoculation or through seed. It can be transmitted by several aphid species, namely *Aphis craccivora*, *A. gossypii*, *M. euphorbiae* and *M. persicae* (Grylls & Butler, 1956; Smith, 1966). However *A. craccivora* is the most important and efficient vector in transmission of SCSV. Vector specificity varies among isolates of the virus. For example, *M. persicae* transmitted isolates from Canberra and Victoria but it did not transmit some isolates from Tasmania (Johnstone & Patten, 1981).

Properties of virus particles

On the basis of some characteristics of the disease, it was suggested that the causal virus may be a member of the luteovirus group (Rochow & Duffus, 1981). However, Chu and Helms (1988)

demonstrated that in plants with symptoms of subterranean clover stunt are virions that are representative of a new group of plant DNA viruses which are isometric, 17 to 19 nm in diameter and sediment as a single component with a buoyant density of 1.24g/ml in Cs_2SO_4. The A_{260nm}/A_{280nm} of purified preparations is about 1.35 which is consistent with an estimated nucleic acid content of 17%.

Ecology of SCSV and its vector

A. craccivora is the most important vector of SCSV. Its ecology has been studied in detail and a model was developed to explain the migration of the aphid and SCSV (Gutierrez *et al.*, 1971 ; Gutierrez *et al.*, 1974a, 1974b). Climate influences development and movement of cowpea aphid populations in eastern Australia. The aphid can not tolerate cold conditions and has a relatively high minimum temperature threshold of 8.3°C for development. It overwinters on small woolly burr medic (*Medicago minima*) and burr medic (*Medicago polymorpha*) in south-eastern Queensland and northern New South Wales (Johnson, 1957; Gutierrez *et al.*, 1974b). These alates may migrate more than 1000km in wind currents exploiting legumes along eastern Australia as they disperse, and causing sporadic outbreaks of SCSV in the central tablelands of New South Wales (Grylls, 1972), in Victoria (Smith, 1966) and in Tasmania (Wade, 1957). The populations of aphids retrace northwards from south-eastern Australia in autumn (Johnstone & McLean, 1987). In Western Australia, SCSV was found to be more common in years following a high summer rainfall (Shipton, 1967), presumably because it helped more aphids survive the summer period.

1.4 Dependent transmission of viruses by aphids

Plants are often found naturally infected with more than one virus. Bennett (1953) reported that curly top, mosaic, yellow net and dodder latent viruses frequently occurred together in sugar beet (cited by Rochow, 1972). These mixed infections may involve viruses that are transmitted by aphids together with others that are not aphid transmissible. The non-aphid transmissible viruses may become transmissible by aphids when another virus or virus product (known as a helper virus, assistor virus, helper agent or helper component) is also present (Freitag, 1969; Falk & Duffus, 1981). There have been many reports on the dependent transmission of plant viruses by aphids, including ones that have non-persistent relationships with their vectors and others that have persistent relationships (Rochow, 1977).

Helper factors associated with the dependent transmission of non-persistent viruses by aphids

Dependent transmission of a non-persistent virus by aphids involving potato aucuba mosaic virus (PAMV) was first reported in 1936 by Clinch *et al.* (cited by Pirone, 1977). PAMV was found to be transmissible by mechanical inoculation, but it could not be transmitted from potato plants using *M. persicae* unless the plants were also infected with potato virus A (PVA). The helper and dependent viruses did not have to be present in the same plants; Kassanis and Govier (1971a) found that PAMV and potato virus C (PVC) were transmitted by aphids from plants infected with them alone if the aphids had fed or probed beforehand on plants infected with potato virus Y (PVY). The component produced in the leaves of plants infected with the helper virus has been termed

the "helper component" (HC).

Most of the non-persistent viruses known to be dependent upon HC sources from infected plants before becoming aphid transmissible are in the potyvirus group. PVC and non-aphid transmissible isolates of peanut mottle virus and tobacco etch virus (Kassanis & Govier, 1971b ; Simons, 1976 ; Pirone, 1977). Similarly, the Campbell isolate of cauliflower mosaic virus, a member of the caulimovirus group, was only transmitted by *M. persicae* if the aphids had fed beforehand on sources of the aphid-transmissible cabbage B isolate of cauliflower mosaic virus (Lung & Pirone, 1973).

The aphid transmissibility of potyviruses and caulimoviruses is commonly lost after the purification process, hence these purified viruses also need a HC source produced in infected plants (Pirone & Harris, 1977). For example, transmission of cauliflower mosaic virus (CaMV) and a number of potyviruses from infectious purified preparations by aphids only occurs if the vectors have simultaneous or prior access to plants or preparations containing appropriate HC (Govier & Kassanis, 1974b; Kassanis & Govier, 1971b; Lung & Pirone, 1974).

Not all groups of non-persistent viruses require HC's for transmission by aphids from the purified preparations. For example, alfalfa mosaic virus (AMV) and cucumber mosaic virus (CMV) are readily transmitted in this way (Pirone & Megahed, 1966), either because they have a HC that is not lost during purification, or because they are firmly bound to the virus particles (Pirone, 1977).

HCs produced in plants infected with several different potyviruses may aid the transmission of other potyviruses by aphids

although their effectiveness may vary. For example, PAMV became aphid transmissible in the presence of HC from plants infected with either BYMV, PVA, PVY or henbane mosaic virus (HMV) but HC from plants infected with PVY was a more efficient source (Kassanis & Govier, 1971b).

HCs in plants infected with potyviruses can not serve as a source of helper for the dependent transmission of non-aphid transmissible isolates of viruses in other groups such as CMV, CaMV or tobacco mosaic virus (Pirone, 1977). Similarly, HC in plants infected with CaMV failed to assist in aphid transmission of purified potyviruses (Lung & Pirone, 1974).

The specificity of HC-virus interactions varies with the viruses and strains responsible for the production of HC and with the dependent viruses themselves (Pirone & Thornbury, 1983). For example, ten strains of PAMV were reported by Kassanis (1961) to be transmitted by *M. persicae* when present in plants infected with either PVA or PVY, but two other strains were aided only by PVY.

Sako and Ogata (1981a) showed that the HCs associated with different potyviruses were significantly different. Watermelon mosaic virus-2 (WMV-2) HC enabled *M. persicae* to transmit TuMV but not PVY, PVY HC served as a helper for *M. persicae* transmission of TuMV but not WMV-2, and TuMV HC did not assist in the transmission of WMV-2 or PVY by *M. persicae*. These results led Sako and Ogata to conclude that the interactions between potyviruses and their HCs are very specific although the HCs induced by some potyviruses have the ability to serve as HCs for some other members of this virus group.

The properties of the HC have been studied in considerable detail, especially for PVY HC (Harris, 1983). The HC remains in the supernatant after centrifuging the extracts sufficiently to sediment

potyviruses. The PVY HC is a protein with a molecular weight between 100,000 and 300,000 daltons. It is serologically distinct from the viral coat protein and the inclusion body protein (Govier *et al.*, 1977; Pirone, 1977). The biological activity of HC's in sap extracts is unstable and is lost by incubation for several hours at 25°, for one day at 4° or for five minutes at 55° (Govier & Kassanis, 1974b ; Sako & Ogata, 1981b). Loss of activity in extracts can be reduced by the addition of Na-DIECA, Na-EDTA or Triton X-100 (Sako, 1980). The chelating agents seem either to protect the HC from enzymatic hydrolysis or else to prevent the HC from binding to insoluble components in the extracts (Hiebert & McDonald, 1973).

Dependent transmission of non-persistent viruses occurs not only when aphids feed or probe on plants infected with both dependent and nondependent viruses, but also when they first feed on sources of suitable HC's before having access to the dependent viruses (Pirone, 1977). This feature suggested that the HC might act by allowing the dependent virus to bind to receptor sites on the aphids' stylets from which it can later be released (Govier & Kassanis Govier, 1974b). Evidence in support of this theory was provided by Berger & Pirone(1986) who found that, in the presence of HC, ^{125}I-labelled potato virus Y virions were associated with the maxillary stylets and with portions of the alimentary canal anterior to the gut whereas label accumulated only in the gut in aphids not given access to HC.

Luteoviruses dependent on other luteoviruses for transmission by aphids.

Mixed infections between luteoviruses have been commonly found in nature. For example, beet western yellows and soybean dwarf viruses often occur together as mixed infections, particularly in

legumes (Johnstone & Duffus, 1984). However the only known examples of dependent aphid transmission from mixed luteovirus infections have involved mixtures of various barley yellow dwarf viruses. Mixtures of barley yellow dwarf viruses are also common in nature (Rochow & Muller, 1974 ; Guy *et al.*, 1987).

Viruses causing barley yellow dwarf (BYD) are among the most important affecting cereals, causing stunting and chlorosis or leaf reddening on small grain cereals and grasses (Rochow, 1970a).

Five distinct viruses causing BYD have been characterized in North American studies. They are distinguished on the basis of their vector specificities and serological reactions (Gill, 1969; Rochow, 1970a). Four of them, referred to as MAV, RMV, RPV and SGV, are transmitted specifically by *Macrosiphum avenae*, *Rhopalosiphum maidis*, *R. padi* and *Schizaphis graminum* respectively. The fifth serotype, designated PAV, is transmitted non-specifically by several aphid species including *M. avenae* and *R. padi*. It normally causes more severe symptoms than the other four serotypes (Rochow, 1970a).

These five viral serotypes can be separated into two groups based on serological tests such as double diffusion in agar gels (Aapola & Rochow, 1971), ELISA using polyclonal (Rochow & Carmichael, 1979) or monoclonal (Diaco *et al.*, 1986) antibodies, cytopathological effects (Gill & Chong,1979) or nucleic acid hybridization analyses (Waterhouse *et al.*, 1986). The RMV and RPV serotypes form one group while MAV, PAV and SGV form the other.

Although the five serotypes of BYD mentioned above are highly vector-specific, some of the serotypes can serve as helpers for other serotypes in aphid transmission. *R. padi* does not normally transmit MAV from singly infected oat plants but will transmit MAV when it is present together with the *R. padi* specific RPV serotype

as a mixed infection (Rochow, 1970b). Similarly, *R. maidis* which is unable to transmit MAV by itself can transmit this virus when it is present together with the *R. maidis* specific RMV serotype in doubly-infected plants (Rochow & Muller, 1974; Rochow, 1975). The vectors are specific for each helper virus, for example *R. padi* does not transmit MAV from plants infected by RMV and MAV, and *R. maidis* can not transmit MAV from mixed infections with RPV and MAV (Rochow, 1975). Several reports of dependent transmission by aphids of various mixed serotypes of BYD indicated that PAV, RMV and RPV could act as helper viruses in independent virus transmission systems. The RPV serotype is the most effective in this regard because it serves as a helper for the transmission of MAV, RMV and SGV by *R. padi*. The PAV serotype is more effective in enabling *R. padi* to transmit MAV than it is to transmit RMV. The RMV serotype is the least effective among the three helper viruses because it only serves as a helper for MAV and RMV in transmission by *R. maidis* (Rochow, 1970b; 1975; 1982).

"Heterologous encapsidation" is a term devised by Rochow (1977) to describe the enclosure of the nucleic acid of one virus within a protein capsid comprised either partly or wholly of protein subunits of a second virus. Rochow (1972, 1977) suggested that phenotypic mixing and transcapsidation should be used to describe two alternate types of heterologous encapsidation as illustrated in Figure 1.1.

Heterologous encapsidation commonly occurs in mixed infections of both bacteriophages and animal viruses. It can occur not only between related viruses but also between distinct, unrelated viruses (Rochow, 1977). Although heterologous encapsidation is also known to occur in plants, little work has been undertaken on this aspect.

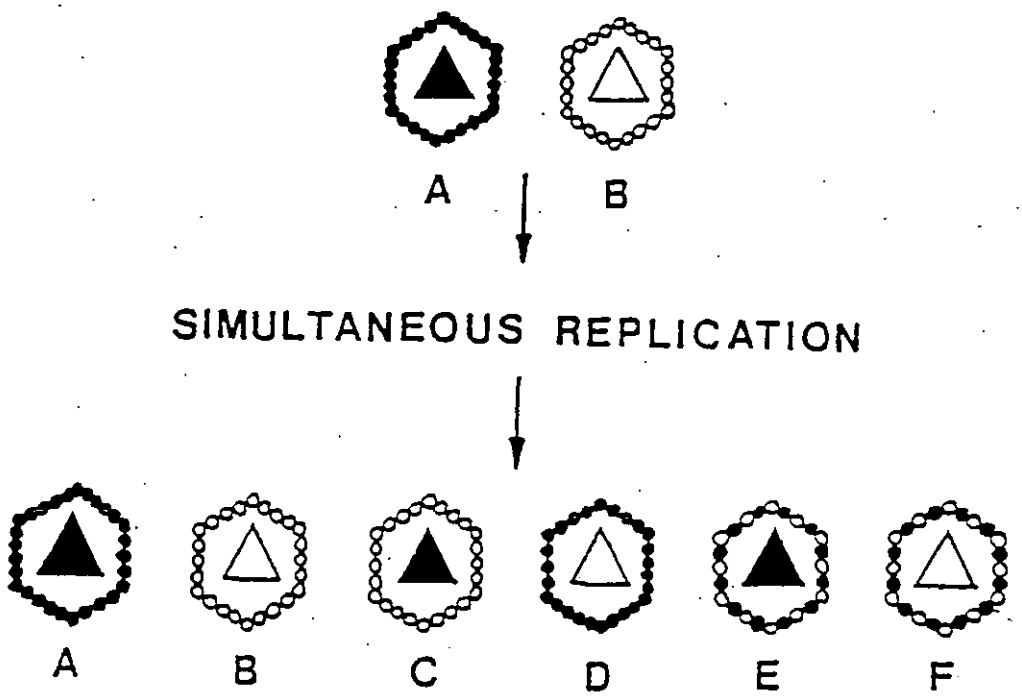


FIGURE 1.1

Possible combinations of nucleic acid and protein capsids for two viruses (A,B) replicated in a mixed infection. Six possible kinds of particles produced in the infection include two (A,B) identical to the viruses used to originate the mixed infection, two (C,D) that illustrate transcapsidation, and two (E,F) that illustrate phenotypic mixing (Rochow, 1977).

There is a variety of possible roles of heterologous encapsidation involving inter-relationships between heterologously encapsidated viruses, their aphid vectors and their host plants. Studies on dependent virus transmission of BYD serotypes by aphids from mixed infections have demonstrated that the viral nucleic acid codes for changes in metabolism in plant cells result in disease symptoms. However the aphid vectors of these plant viruses only recognize the coat protein of the transmissible virus. Heterologous encapsidation thus provides a mechanism for the dependent transmission of persistent luteoviruses by aphids (Rochow, 1977). It might lead to the creation of new diseases as a result of extended host ranges if the transient vector of the dependent virus has a different set of host feeding preferences. For example, virus particle C (in Figure 1.1) containing nucleic acid of A might become transmitted by an aphid species different from the usual vector of A (Rochow, 1977).

It is known that *R. padi* does not regularly transmit MAV from singly-infected plants but that *R. padi* will frequently transmit MAV together with RPV from a mixed infection of the two viruses. There was no evidence for dependent transmission of MAV when *R. padi* fed first on a source of either MAV or RPV and then later on a source of the alternate virus irrespective of whether it was acquired by feeding on infected leaves, by feeding through membranes on purified virus preparations or by micro-injection of virus into the haemocoele of aphids. Only RPV was transmitted in these experiments (Rochow, 1970b, 1979). Dependent transmission of luteoviruses only occurred when aphids fed on doubly-infected plants. The phenomenon therefore appears to result from interaction of the dependent virus and the helper virus in their common host plant and presumably

involves heterologous encapsidation (Harris, 1979). This is in contrast to the dependent transmission of non-persistent viruses by aphids where the dependent virus may be transmitted not only from plants with a mixed infection but also when aphids feed first on a source of helper component and then later on the dependent virus (Pirone, 1977).

Studies on dependent transmission of MAV by *R. padi* from mixed infections with RPV led to the first evidence for transcapsidation in mixed plant virus infections. Since MAV and RPV are serologically unrelated viruses, it was possible using serological techniques (infectivity neutralization) to demonstrate the occurrence of heterologous enscapsidation in the mixed infection situation. When purified preparations made from mixed infections of MAV and RPV were treated with MAV antiserum and then fed to aphids through membranes, *M. avenae* did not transmit either virus because all the MAV coat protein encapsidating either MAV RNA or RPV RNA in the preparation had been neutralized by the MAV antiserum. On the other hand, *R. padi* could transmit both MAV and RPV from the same preparations. Thus, virus particles in a mixed infection made up of MAV RNA encapsidated in RPV protein coat seem to function in *R. padi* like RPV (because of the RPV protein) and in the plant like MAV (because of the MAV nucleic acid) (Rochow, 1977).

CHAPTER 2

General Experimental Procedures

2.1 Virus isolates

The legume viruses and strains used in the experiments were isolated from plants collected in different regions of Tasmania (Table 2.1). The viruses were identified using host range, aphid transmission and serological tests, including enzyme-linked immunosorbent assays (ELISA). Each virus isolate was maintained in appropriate plant species in an insect-screened glasshouse. Bean yellow mosaic virus isolates were mechanically inoculated into *Vicia faba* cv. Coles Dwarf Prolific while the two isolates of clover yellow vein virus were maintained in *Trifolium subterraneum* cv. Mt. Barker. *Nicotiana glutinosa* was used as the propagation host for cucumber mosaic virus. Serial transfers with aphids were used to maintain cultures in *T. subterraneum* cv. Mt. Barker of the two soybean dwarf virus isolates (SDV-Ap and SDV-As) and subterranean clover stunt virus using *Acyrthosiphon pisum*, *Aulacorthum solani* and *Aphis craccivora* respectively. Isolates of SDV transmitted specifically by *A. pisum* had not been recorded previously in Australia. Acquisition access and inoculation feeding periods were each three days. The symptoms of the virus isolates on *T. subterraneum* and *V. faba* are shown in Figures 2.1 - 2.5.

2.2 Plants

All the plants used to maintain the virus isolates and virus-free aphid cultures and to characterize the virus properties were grown in an artificial soil mix. It consisted of 1:1, peat moss: coarse sand with a broad fertilizer dressing of 500g ammonium-

TABLE 2.1

Sources of the viruses.

Virus	Host species	Location	Acronym
bean yellow mosaic	*Vicia faba*	Forth	BYMV-F
bean yellow mosaic	*Trifolium subterraneum*	Kempton	BYMV-K
clover yellow vein	*Vicia faba*	Burnie	CYVV-B
clover yellow vein	*Vicia faba*	Devonport	CYVV-D
cucumber mosaic	*Trifolium subterraneum*	Sandford	CMV
soybean dwarf (syn. subterranean clover red leaf)	*Trifolium subterraneum*	Cambridge	SDV-Ap
soybean dwarf	*Trifolium repens*	North West Coast	SDV-As
subterranean clover stunt	*Vicia faba*	Hagley	SCSV

(a)

(b)

FIGURE 2.1

Symptoms of BYMV on *Trifolium subterraneum* (a) and *Vicia faba*(b)

(a)

(b)

FIGURE 2.2

Symptoms of CMV on *T. subterraneum* (a) and *V. faba* (b)

(a)

(b)

FIGURE 2.3

Symptoms of CYVV on *T. subterraneum* (a) and *V. faba* (b)

(a)

(b)

FIGURE 2.4

Symptoms of SCSV on *T. subterraneum* (a) and *V. faba* (b)

(a)

(b)

FIGURE 2.5

Symptoms of SDV on *T. subterraneum* (a) and *V. faba* (b)

nitrate, 200g potassium sulphate, 300g Micromax^{R} (a proprietary mixture of balanced plant micronutrients) , 1500g superphosphate and 3000g dolomite per cubic metre. Normal Hoaglands solution (Hewitt 1966) was watered into the mix once or twice weekly as necessary.

The plants were grown in insect screened glasshouses (Figure 2.6) and were fumigated weekly with CIG Insectigas D (50 g/kg dichlorvos) at a rate of 0.67g/m^3.

2.3 Aphids

Each virus-free aphid species was maintained separately as virus free colonies in cages (36 x 40 x 60cm or 30 x 60 x 60cm) which consisted of Terylene voile and perspex with an aluminium frame (Figure 2.7). Host plants used for the various aphid species are listed in Table 2.2. The plants in the cages were renewed weekly. The identity of each culture was confirmed as correct by Dr. V.F. Eastop, British Museum, London.

For aphid transmission of the non-persistent virus isolates young nymphs were taken from the cultures and fasted for 3 - 4 hours at room temperature or 24 hours at 4°. Subsequently, the aphids given acquisition access periods (AAP) of 3- 5 minutes on infected leaves in Petri dishes and they were then transferred onto healthy test plants (Figure 2.8). After 24 hours the aphids were killed by fumigating three times with maldison (Malathion 5 ml/3.5 l) (1 - 2 min.) at 1 - 2 hour intervals using Fogmaster Tri-Jet (Challenger) Model 6208 (Figure 2.9). The plants were kept in the room overnight before returning them to the glasshouse.

The persistent virus isolates were transferred using either single aphids or groups of young nymphs from the aphid cultures. The aphids were moved onto young healthy test plants after a three

TABLE 2.2

The host plants on which the aphid species were reared.

Aphid species	Host plant
Acyrthosiphon kondoi	*Lens culinaris*
Acyrthosiphon pisum	*Vicia faba*
Aphis craccivora	*Vicia faba*
Aulacorthum solani	*Datura stramonium* & *Geranium dissectum*
Cavariella aegopodii	*Anethum graveolens*
Hyperomyzus lactucae	*Sonchus oleraceus*
Macrosiphum euphorbiae	*Sonchus oleraceus*
Myzus persicae	*Raphanus sativus*
Rhopalosiphum padi	*Avena sativa*

FIGURE 2.6

Glasshouse where plants were propagated and inspected for symptoms of virus infection.

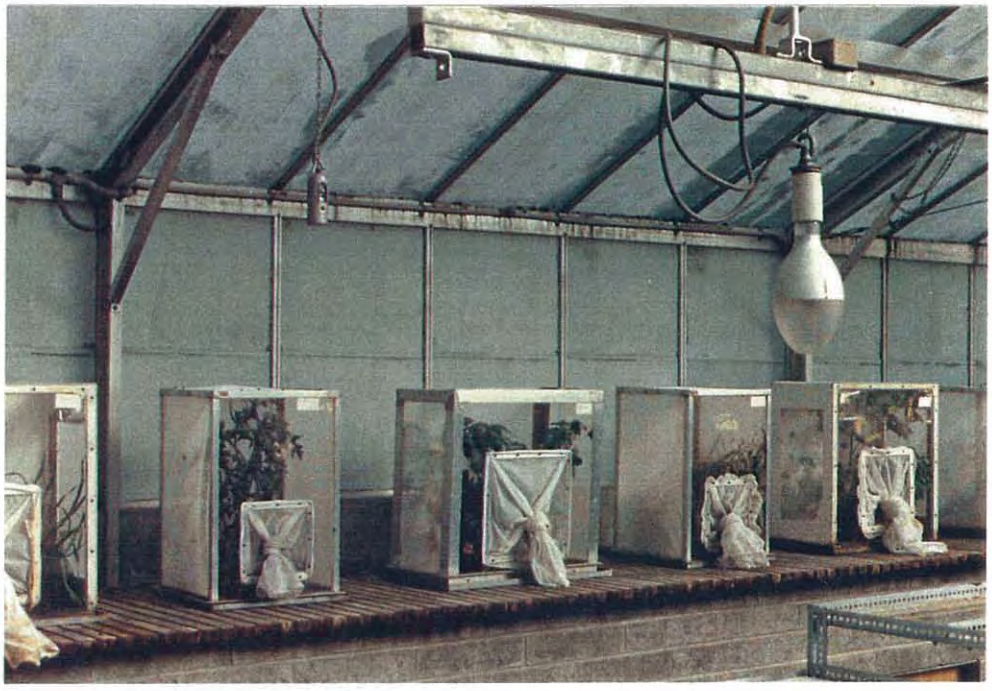


FIGURE 2.7

Culture cages for rearing virus-free aphids in controlled glasshouse conditions.

FIGURE 2.8

Caption Aphids after acquisition access to virus sources caged on young healthy test seedlings.

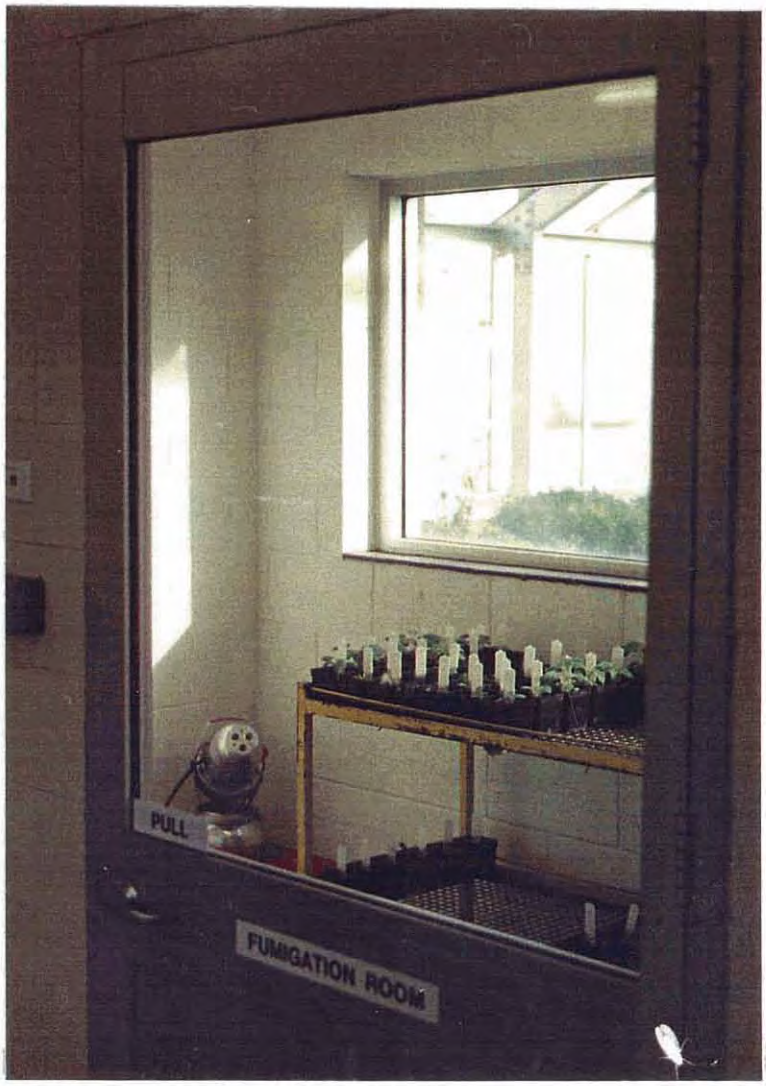


FIGURE 2.9

Aphids were killed in the fumigation room before returning the plants to the glasshouse.

day acquisition access feeding period. Three days later the plants were fumigated as outlined above.

Aphid trapping and identification

Aphid flight activity was monitored using Moericke traps. They consisted of yellow plastic trays (29 x 33 x 12cm) filled with water containing 0.5% Teepol^{R} (a proprietary industrial detergent) at a height of 1.5 m above ground level. Two traps were placed at the University Farm, Cambridge adjacent to the sites of the field experiments and two others were set up at Sandford, 18 km away (see Figure 3.1). The traps were normally cleared weekly. All aphids were carefully removed from the trays and preserved in plastic vials containing a mixture of 75% lactic acid and 90% ethanol (ratio 1:2) pending identification.

Each aphid was examined under a stereomicroscope and identifed using the keys of Martyn (1960) and Taylor (1984).

2.4 Mechanical inoculation

Infected leaves were ground in 0.1 M phosphate buffer pH7.5 containing 0.01 M L-cysteine (1:3, w:v) in chilled mortars. Before inoculation the leaves were dusted lightly with 500-mesh carborundum (silicon carbide). The ground virus inoculum was gently rubbed over the leaf surfaces using either a finger or pad of cotton gauze dipped in the inoculum. The leaves were washed with water before returning the plants to the glasshouse.

2.5 Electron microscopy

Grids (300-400 mesh) were coated with 2% collodion or Parlodion (nitrocellulose) and then with carbon in a vacuum

evaporation chamber (Dynavac). Samples were stained with potassium phospho-tungstate (1-2% w/v, pH 7.0) for examination of extracts from fresh plant samples or uranyl acetate (2% w/v) for examination of purified virus preparations. For fresh plant samples a small piece of leaf tissue was macerated on a glass slide in a drop of stain using a metal dissecting needle. A carbon coated grid held with a pair of forceps was floated on the macerate for 1-2 minutes and then drained by touching the edge of the grid with a small piece of filter paper. For purified viruses, carbon coated grids were floated for 2-5 minutes on dilutions of the preparations placed on pieces of Parafilm in Petri dishes. The grids were then washed with twenty drops of distilled water from a Pasteur pipette. Next, about six drops of uranyl acetate were washed over the grids which were then drained as before.

2.6 Serological tests

The methods used for double antibody sandwich ELISA (DAS-ELISA) were based on the procedures described by Clark and Adams (1977). Immunoglobulins were purified from the antisera by precipitation with half-saturated ammonium sulphate, resuspended in half-strength phosphate-buffered saline (PBS), dialysed against half-strength PBS and eluted through columns of Whatman DE 22 diethylaminoethyl cellulose. The purified IgG was adjusted to a final concentration of 1mg/ml (O.D280nm = 1.4), preserved by adding sodium azide to 0.01% and stored in silicone-coated tubes at 4°. Aliquots (0.8ml) of the purified IgG were conjugated with 2.0mg alkaline phosphatase in 0.06% glutaraldehyde at room temperature for 4 hours. After dialysis against PBS, bovine serum albumin (5mg/ml) was added to the conjugate solutions which were then stored at 4° in

silicone-coated tubes.

DAS-ELISA tests were performed in polystyrene microtitre plates using volumes of 200µl per well (Clark & Adams, 1977). The wells were first coated with purified IgG (1µg/ml in carbonate buffer, pH 9.6) and incubated at 4° for 24 hours. The plates were then rinsed three times with PBS-Tween (PBS containing 0.05% Tween 20). Plant samples ground in extracting buffer (1:5, w:v) were centrifuged for 2 minutes at 5000g and incubated in the coated plates at 37° for 4 hours. After rinsing, conjugated IgG was added (1µg/ml) and the plates incubated overnight at 4°. After rinsing the wells, the bound IgG-enzyme conjugate was detected by adding 200µl of *p*-nitrophenyl phosphate (0.6mg/ml). Absorbances were read in a Titertek Multiskan^{R} photometer at 405nm after the reactions had been stopped with 50µl of 6M NaOH, generally after 1 - 2 hours of hydrolysis.

Extracts from healthy plants, buffer controls and positive controls were included in each plate.

Indirect ELISA tests involved treating purified IgG preparations with pepsin to cleave them and produce virus-specific $F(ab')_2$ fragments (Barbara & Clark, 1982). Polystyrene microtitre plates were coated with these $F(ab')_2$ fragments diluted in carbonate coating buffer by incubating them overnight at 4°, followed by the virus samples for 4 hours at 37°, then varying dilutions of whole purified IgG overnight at 4°, protein A conjugated to alkaline phosphate for 4 hours at 37°, and finally with the alkaline phosphatase substrate *p*-nitrophenyl phosphate (0.6mg/ml). The plates were rinsed three times with PBS-Tween between each step.

Another indirect ELISA test involved coating plates with leaves extracted in carbonate coating buffer (pH 9.6) and detecting

the virus bound to the wells with a specific IgG, followed by incubation with an anti-animal IgG enzyme conjugate (Jordan & Hammond, 1986).

Immune electron microscopy was used to distinguish viruses using the modified Derrick method (Milne & Luisoni, 1977). Grids were floated for 30 minutes at room temperature (c. 25°) on 15 µl drops of antiserum diluted 1:1000 in 0.1M phosphate buffer (pH 7.0) and placed in plastic Petri dishes or on pieces of Parafilm placed in glass Petri dishes. The grids were then washed with 20 consecutive drops of the same buffer and drained of excess moisture by a brief contact with filter paper. The serum-coated grids were next floated for 30-60 minutes on 15µl drops of virus suspensions or extracts of infected tissue. The grids were washed again with 20 consecutive drops of buffer followed by 30 drops of distilled water. Finally, five drops of freshly prepared uranyl acetate were washed over the grids. They were then carefully drained by touching filter paper to the edge of each grid.

CHAPTER 3

Field survey of viruses in *Trifolium subterraneum*, *Vicia faba* and other legume crops in Tasmania.

3.1 Introduction

The first virus reported infecting *V. faba* in Tasmania was bean yellow mosaic virus (syn. pea mosaic virus) (Wade, 1950). Soybean dwarf virus and subterranean clover stunt virus have been recognised in broad bean crops since 1971 (Johnstone, 1978), while clover yellow vein virus was reported to infect broad beans by Munro (1981).

Soybean dwarf virus was reported to cause the greatest virus disease problem in Tasmanian broad bean crops because it was most common and the yields of plants were drastically reduced following early infection (Johnstone & Rapley, 1979). Other viruses infecting *V. faba* on mainland Australia but not recorded on this species in Tasmania include broad bean wilt virus, cucumber mosaic virus and tomato spotted wilt virus (Büchen-Osmond *et al.*, 1988).

Until recently only two viruses had been reported infecting subterranean clover in Tasmania, namely soybean dwarf virus and subterranean clover stunt virus (Sampson & Walker, 1982). The former virus, first recorded in 1968, was noted to be very common whereas subterranean clover stunt, first found in 1950, was reported as uncommon in recent years (Grylls & Butler, 1956 ; Anon., 1968). Beet western yellows virus was subsequently found infecting subterranean clover in Tasmania by Johnstone & Duffus (1984).

A number of other viruses have been recorded infecting

subterranean clover on mainland Australia. These include alfalfa mosaic, bean yellow mosaic, clover yellow vein, cucumber mosaic, subterranean clover mottle and white clover mosaic viruses (Johnstone & McLean, 1987). Most of these viruses are considered to cause sporadic disease outbreaks in localised areas but little published information exists on their incidence in pastures. Sometimes very high levels of virus infection have been found (Helms, 1987 ; McGhee, 1989) but in other cases the levels have generally been low (Garrett, 1987 ; Jones & McKirdy, 1990).

A survey of selected subterranean clover pastures in Tasmania was carried out between 1985 and 1988. In addition, some *V. faba* and other legume crops in north-western Tasmania were sampled and tested in 1986 and 1987 when symptoms of virus infections were found in them during irregular inspections.

3.2 Materials and Methods

Subterranean clover pastures

A total of twelve pastures were selected for inclusion in a regular survey. Two were at Sandford, one at Cambridge, four at Richmond, two at Kempton, two at Campbell Town and one at Cressy (Figure 3.1). All the pastures were a mixture of subterranean clover cvs. Mt. Barker and Woogenellup together with perennial ryegrass (*Lolium perenne*). The pastures were inspected twice each year, once during the autumn/winter period and then again in late October/November, after the peak of clover flowering but before the plants had commenced to senesce. The first inspections were made in October 1985 and the last in November 1988. At each inspection a total of twenty areas 0.5 x 0.5 m were selected at random in each pasture using quadrats and all subterranean clover plants within the

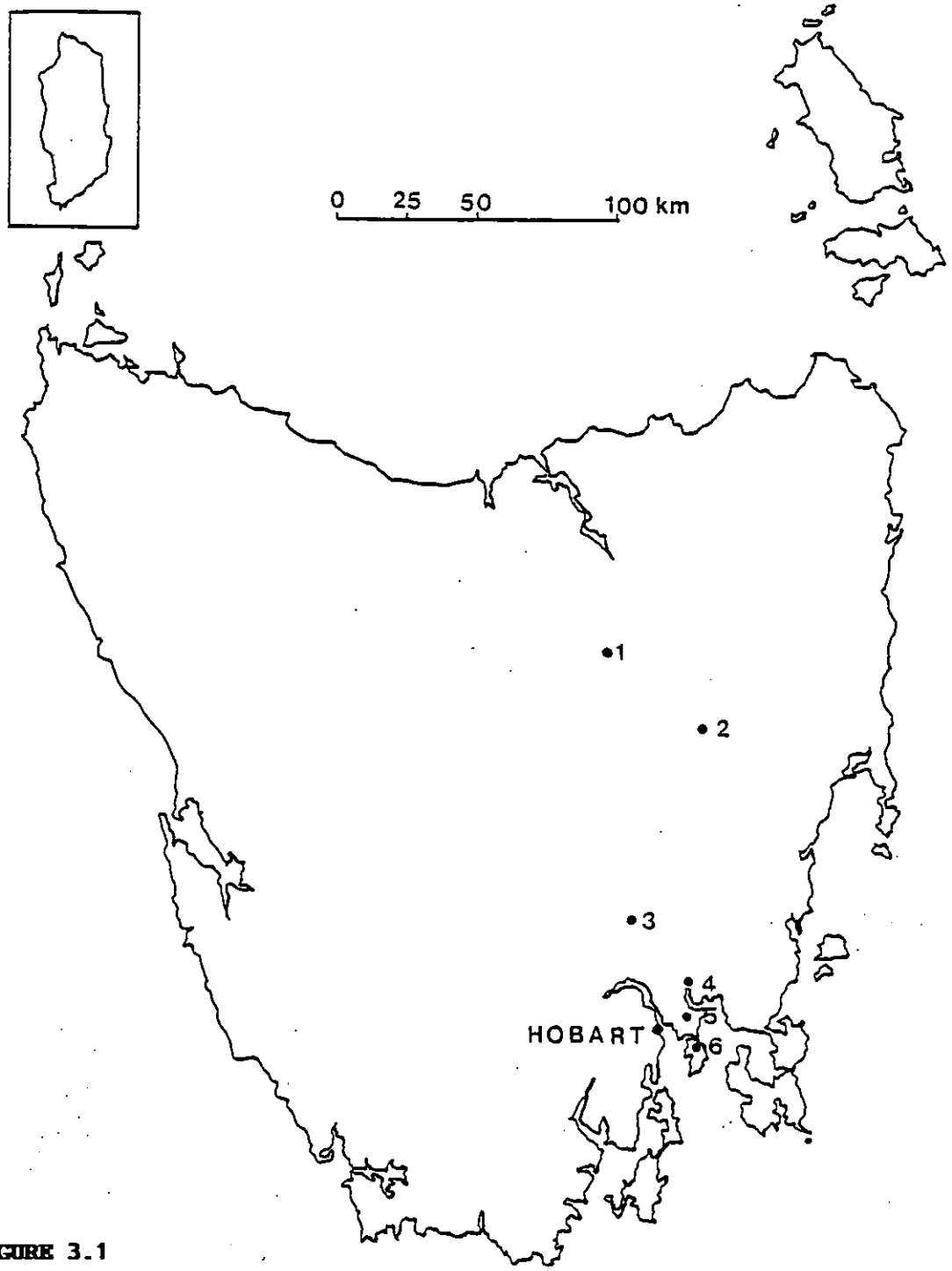


FIGURE 3.1

Locations of subterranean clover pastures surveyed between 1985 and 1988.

Code of sites - 1: Cressy 2: Campbell Town

3: Kempton 4: Richmond

5: Cambridge 6: Sandford

quadrats were examined for viral infections relative to the total number of clover plants present. In addition to the pasture at Cambridge, volunteer subterranean clover occurring as weeds in and around crops on the property where the field experiments were conducted, were inspected at the same times for symptoms of virus infection.

Samples of all plants considered to possibly be infected with a virus were returned to the laboratory for confirmation and identification by either mechanical transmission to indicator plants, aphid transmission to indicator plants or enzyme-linked immunosorbent assay (ELISA). Representative samples of plants found in the field displaying definitive virus symptoms were also taken to confirm the diagnoses.

At the final three inspections a total of twenty clover samples were taken at random from each of the twelve pastures to test for virus infections by ELISA in order to determine whether infected plants might have been missed during the visual inspections because of poor symptom expression. Samples were tested for the presence of alfalfa mosaic, bean yellow mosaic, clover yellow vein, cucumber mosaic, soybean dwarf, subterranean clover mottle and subterranean clover stunt viruses. Positive, negative (healthy plant extracts) and buffer controls were included in duplicate wells in every plate. Absorbances recorded in the ELISA assay were considered positive when they were more than three times the mean of the duplicate wells in which healthy plant sap extracts had been incubated.

Survey of legume crops in north-western Tasmania

Legumes with symptoms like those caused by luteovirus

infections were sampled from crops inspected on the north-western coast of Tasmania in 1986 and 1987. The symptoms included reddening, rolling, thickening and yellowing of the leaves.

Each sample was divided into two parts. The bases of these samples were plunged into about 6ml of 1% water agar in the bottom of 22 mm test-tubes. Aphids were allowed to feed on the samples in the tubes for three days before transferring them to subterranean clover cv. Mt. Barker indicator plants for a three day inoculation access period. *A. solani* were placed in one tube of each pair and transferred to individual indicator plants in groups of about five while *A. pisum* were placed in the other tube and transferred in groups of ten.

V. faba crops at the Forth, near Devonport on the northwestern coast, were also inspected for mottle, mosaic and necrosis symptoms caused by non-persistent viruses (alfalfa mosaic, bean yellow mosaic, cucumber mosaic and clover yellow vein viruses). Samples were taken from plants displaying symptoms and checked for infection with viruses by mechanical inoculation to indicator plants, electron microscopy, and ELISA.

3.3 Results

Subterranean clover pastures

Numbers of plants observed with virus symptoms and proven to be infected are detailed in Tables 3.1 and 3.2. Infection levels were generally low and sporadic. Only one plant was found infected with clover yellow vein virus, at Kempton in the spring of 1986, and mechanical inoculations from this plant caused vein yellowing on subterranean clover and systemic necrosis on *V. faba*.

Plants with symptoms of subterranean clover stunt were found

TABLE 3.1

Numbers of subterranean clover plants found infected with various viruses at each time of inspection.

Year	Time of year	Number of plants examined	Number of plants found infected with						
			AMV	BYMV	CMV	CYVV	SCMoV	SCSV	SDV
1985	Spring	2920	0	9	1	0	0	86	4
1986	Autumn	4185	0	17	0	0	0	0	124
	Spring	2273	0	35	0	1	0	0	3
1987	Autumn	1524	0	0	0	0	0	0	1
	Spring	1705	0	0	0	0	0	0	5
1988	Autumn	2032	0	0	0	0	0	0	0
	Spring	1708	0	0	0	0	0	0	0

AMV alfalfa mosaic virus
BYMV bean yellow mosaic virus
CMV cucumber mosaic virus
CYVV clover yellow vein virus
SCMoV subterranean clover mottle virus
SCSV subterranean clover stunt virus
SDV soybean dwarf virus

TABLE 3.2

Numbers of virus-infected subterranean clover plants found at each site at each time of inspection.

Year	Time of year	Number of plants examined	Number of plants infected at					
			Cambridge	Campbell Town	Cressy	Kempton	Richmond	Sandford
1985	Spring	2920	3	0	0	19	67	11
1986	Autumn	4185	5	0	0	16	119	1
	Spring	2273	1	34	0	4	0	0
1987	Autumn	1524	0	0	0	0	0	1
	Spring	1705	2	1	0	1	1	0
1988	Autumn	2032	0	0	0	0	0	0
	Spring	1708	0	0	0	0	0	0

only once, during the 1985 spring inspections, and then only in the Kempton and Richmond pastures. The pastures at Richmond, each one hectare in area, adjacent to each other and forming part of a grazing experiment had significantly different levels of infection (P=0.05), varying from 0.9% to 18.2%. Similarly the levels of infection in the two Kempton pastures were significantly different (P=0.05) with means of 1.9% and 5.3%.

Infection with soybean dwarf virus was also sporadic and localised. Most infections were found in the Richmond pastures in the autumn of 1986. Levels of infection in the four pastures varied from 0.4 to 18.2 per cent. The infected plants did not persist through to the spring. All the plants found with symptoms of SDV at the time of the 1985 spring inspection were in the Richmond pastures. In the autumn of 1987, only one infected plant was found, at Sandford, but a small number of infected plants were located during the spring of that year in the pastures at Cambridge, Campbell Town, Kempton and Richmond.

Infection with bean yellow mosaic virus had a similar pattern to that of soybean dwarf virus occurrence during the 1986 inspections. In the autumn of 1986, 3.5% of the plants in the pasture at one site at Kempton, on a heavy soil, were infected with this virus while none were found in the other Kempton pasture which was on a light soil. Few of the infected plants survived through to the spring. Likewise in the spring of 1986 all the plants (13.9%) at Campbell Town identified as being infected with bean yellow mosaic virus were in the pasture established on a heavy dark soil while no infected plants were found in the pasture nearby on the light soil. Similarly, at the 1985 spring inspection all the infected plants were found in pastures established on the heavy soil

sites at Kempton (0.7%) and Sandford (22.4%) and none were found in the pastures established on the light soils in these two districts.

Only one plant was seen infected with cucumber mosaic virus during the whole course of the survey while none was found with symptoms of alfalfa mosaic and subterranean clover mottle viruses. However results from ELISA tests on samples collected at the last three times of inspection, indicated low levels of infection with alfalfa mosaic, cucumber mosaic and subterranean clover mottle viruses in symptomless plants in some pastures (Table 3.3).

Many volunteer subterranean clover seedlings with bare ground around them in and around crops at the Cambridge site were found infected with either bean yellow mosaic virus or soybean dwarf virus at times when few infections were detected in subterranean clover plants in the pasture that was inspected nearby.

Survey of legume crops in north-western Tasmania

Soybean dwarf virus isolates transmitted specifically by *A. solani* (SDV-As) were recovered from 178 of the 293 plants that were tested because they displayed symptoms suggestive of luteovirus infection. In the same tests SDV-Ap isolates (transmitted by *A. pisum*) were not detected (Table 3.4). One of the species found infected, *Trifolium polymorpha*, had not previously been recorded as a host of the SDV-As.

Testing of *V. faba* plants with mottle, mosaic or necrotic spot symptoms showed that only bean yellow mosaic virus and clover yellow vein virus naturally infected the bean crops in this region (Table 3.5). Alfalfa mosaic virus and cucumber mosaic virus were not found in the *V. faba* crops.

TABLE 3.3

Comparative results from visual observations and ELISA tests at the last three times of inspection.

Virus	Year	Percent infection estimated by	
		Visual inspection	ELISA
AMV	Spring 1987	0.0	0.0
	Autumn 1988	0.0	0.4
	Spring 1988	0.0	0.4
BYMV	Spring 1987	0.0	0.0
	Autumn 1988	0.0	0.0
	Spring 1988	0.0	0.0
CMV	Spring 1987	0.0	0.0
	Autumn 1988	0.0	0.4
	Spring 1988	0.0	3.3
CYVV	Spring 1987	0.0	0.0
	Autumn 1988	0.0	0.0
	Spring 1988	0.0	0.0
SCMoV	Spring 1987	0.0	0.0
	Autumn 1988	0.0	0.0
	Spring 1988	0.0	0.8
SCSV	Spring 1987	0.0	0.0
	Autumn 1988	0.0	0.0
	Spring 1988	0.0	0.0
SDV	Spring 1987	0.3	0.8
	Autumn 1988	0.0	0.0
	Spring 1988	0.0	0.0

TABLE 3.4

Incidence of two soybean dwarf virus isolates transmitted specifically by *A. solani* (SDV-As) and by *A. pisum* (SDV-Ap) in naturally infected legumes in north-western Tasmania.

Host	Number of plants collected	SDV-Ap	SDV-As
Pisum sativum	12	0	6
Trifolium fragiferum	2	0	0
Trifolium hybridum	2	0	2
Trifolium polymorpha	2	0	2
Trifolium repens	8	0	4
Trifolium sempilosum	1	0	0
Trifolium subterraneum	14	0	12
Vicia faba	250	0	152
Vicia pencultanium	2	0	0

TABLE 3.5

Recovery of non-persistent viruses from *Vicia faba* plants at Forth in 1986 and 1987.

Year	Number of plants collected	AMV	BYMV	CMV	CYVV
1986	20	0	14	0	5
1987(a)	12	0	5	0	7
1987(b)	20	0	11	0	8

(a) Samples collected from plots sown in June
(b) Samples collected from plots sown in September

3.4 Discussion

The data obtained during the survey suggested that viruses were exerting little effect on the productivity of subterranean clover pastures over the period of this study. However there was obviously potential for sporadic serious virus epidemics in localised areas as exemplified by the outbreaks of subterranean clover stunt and soybean dwarf in 1985 and 1986 respectively. These viruses are transmitted specifically by different aphid species and the occurrence of the viruses in the pastures reflected aphid activity.

The reasons for the differences in virus incidence in the four pastures at the Richmond site were not clear. The alightment of alate aphids on plants is a complex phenomenon. Colour contrasts play an important role in the attraction of aphids to plants (Kring, 1972) and it is probable that different grazing intensities in the Richmond pastures at the time viruliferous aphids were flying were responsible for the large differences in virus incidence that were recorded. Similarly the differences in virus incidence between the pastures at Campbell Town and Kempton on dark heavy soils compared to those on the light sandy soils possibly reflected attraction of winged aphids to plants with suitable contrasting earth about them. This phenomenon could also explain why many more subterranean clover plants occurring as volunteer weeds on the Cambridge property were infected with viruses than the plants present in the pasture sward nearby.

The ELISA test results indicated the occurrence of small numbers of infections with alfalfa mosaic, cucumber mosaic and subterranean clover mottle viruses in the subterranean clover pastures that were not detected by visual inspection. It was not

clear whether these additional infected plants had only recently become infected, were symptomless as a result of seed-borne infection, or had recently lost their infected leaves as a result of grazing. A few plants additional to those found infected with soybean dwarf virus by visual inspection were also detected by ELISA.

The density of subterranean clover plants in most of the pastures was low. A total of 2000 plants examined at any inspection time (Tables 3.1, 3.2) represented 33 plants per square metre. There are very large seed reserves in Tasmanian subterranean clover pastures (P. Evans, personal communication) but the mild climatic conditions over the pastoral regions of the State are not conducive to breaking hard-seededness and dormancy.

Most isolates of soybean dwarf virus infecting legumes in north-western Tasmania were SDV-As which was in marked contrast to most of those recovered in south-eastern Tasmania which were transmitted specifically by *A. pisum* (see Chapter 6). White clover (*Trifolium repens*) is the predominant legume of pastures in north-western Tasmania, where rainfall is moderately high, and serves as a reservoir of infestation with *A. solani* and infection with SDV-As (Johnstone, 1978). In the south-eastern region of Tasmania, rainfall is low (mean *c.*550mm) and white clover does not persist in the subterranean clover based pastures. The major perennial legume in this region is lucerne (*Medicago sativa*), present in irrigated stands and this is a host of both *A. pisum* and SDV-Ap whereas it is not a host of SDV-As or *A. solani*.

CHAPTER 4

Biological properties of selected potyviruses and luteoviruses

4.1 Introduction

The biological properties of viruses can be useful in classifying strains of viruses because they often vary with respect to properties such as host ranges and vector specificities. Different isolates of both BYMV and CYVV may vary in both host range (Jones & Diachun, 1977) and vector specifity.

Differences in host range and vector specificity of luteoviruses have been important factors in distinguishing members of this group . *A. solani* was generally found to be the most efficient vector of SDV but some isolates were also transmitted by other species (Wilson & Close 1973 ; Ashby *et al.*, 1979 ; Helms *et al.*, 1983). SDV has minimal acquisition access (AAP) and inoculation access periods (IAP) of 30-60 minutes; optimal AAP and IAP were 24-48 hours (Tamada, 1970 ; Damsteegt & Hewings, 1987). Kellock (1971) reported that the minimum AAP and IAP of SCRLV were 6 hours and 20 minutes respectively. Host ranges of various SDV isolates transmitted specifically by *A. solani* included legumes and non-legumes and again, different isolates varied with respect to their abilities to infect some species (Johnstone *et al.*, 1984a). There is only one very brief report on transmission of an SDV isolate by *A. pisum* (Johnstone *et al.*, 1984b).

The aims of the studies reported here were to investigate the biological properties of selected virus isolates from subterranean clover and broad bean, particularly with respect to their host

reactions, aphid vector specificities, and the ways in which they were dependently transmitted by aphids from mixed infections.

4.2 Materials and Methods

Virus isolates

Two isolates of each of BYMV, CYVV and SDV were compared biologically to determine some of their properties. A list of the virus isolates, their original sources and the maintenance host plant species are detailed in Chapter 2.1.

Differential hosts

Plant species tested to check host reactions to the BYMV and CYVV isolates were inoculated mechanically (see Chapter 2.4) and generally indexed after 3-4 weeks by back inoculation to *V. faba* cv. Coles Dwarf Prolific and tested by DAS-ELISA tests using antisera produced to BYMV-F and CYVV-D (see Chapter 2.6). At least five plants of each test plant species (or cultivar) were used for each virus isolate.

The host ranges of SDV-Ap and SDV-As and the symptoms they caused were compared following attempted transmissions using aphids. Young nymphs of *A. pisum* and *A. solani* were allowed to acquire SDV-Ap and SDV-As from infected plants and then transferred to the test plants, *A. pisum* in groups of ten and *A. solani* in groups of five. The acquisition and inoculation feeding periods were each of three days. After 4-6 weeks all the test plants were visually checked for infection and by DAS-ELISA (see Chapter 2.6).

Aphid transmission

a) Potyvirus isolates

Nine aphid species collected in Tasmania were maintained separately as virus-free colonies as described previously in Chapter 2.3. Six of the species were known to colonise broad bean plants in Tasmania, namely *Acyrthosiphon kondoi*, *Acyrthosiphon pisum*, *Aphis craccivora*, *Aulacorthum solani*, *Macrosiphum euphorbiae* and *Myzus persicae*. The other three species *Cavariella aegopodii*, *Hyperomyzus lactucae* and *Rhopalosiphum padi* were common on fennel (*Foeniculum vulgare*), sowthistle (*Sonchus oleraceus*) and barley (*Hordeum vulgare*) respectively, growing near the experimental sowings of broad beans in 1986 and 1987. These nine species were used to compare the vector specificities of the non-persistent BYMV and CYVV isolates. Young nymphs from each aphid culture were starved for 24 hours at 4°. Subsequently, the aphids given acquisition access three to five minutes to *V. faba* (cv. Coles Dwarf Prolific) leaves infected with the various virus isolates in Petri dishes and then transferred to healthy *V. faba* plants for 24 hours. They were then sprayed with an insecticide in the fumigation room before their return to the glasshouse (see Chapter 2.3). The experiments were repeated four times. Each time, each aphid-isolate combination involved ten aphids per plant and ten plants per treatment. The probability of disease transmission by single aphids was estimated from the following formula used by Swallow (1985).

$$(1 - p)^k = H$$

p = the probability of disease transmission by a single aphid.

k = the number of aphids transferred to each test plant.

H = the observed proportion of healthy, uninfected test plants.

b) SDV-Ap

SDV-Ap was originally isolated from an infected subterranean clover plant at the University Farm, Cambridge, in January 1986 (Chapter 6). Detailed information was obtained on the virus-vector relationships of this new isolate of SDV as follows:

i) Comparison of aphid species as vector of SDV-Ap

Young nymphs from colonies of *A. kondoi*, *A. pisum*, *A. solani*, *A. craccivora*, *M. euphorbiae* and *M. persicae* were tested for their ability to transmit SDV-Ap. Subterranean clover (cv. Mount Barker) plants were used as virus sources and as test plants. Each test was done in duplicate using ten aphids per plant. Acquisition and inoculation access periods were each of three days.

ii) Effect of number and morph of *A. pisum* on virus transmission.

Different morphs (young nymphs, adult apterae and adult alatae) of *A. pisum* were allowed to feed on infected plants for 72 hours and then placed singly or in groups of five or ten on healthy subterranean clover seedlings for 72 hours. Subsequently, the aphids were killed with insecticide in the fumigation room.

iii) Persistence of SDV-Ap in *A. pisum*.

Young nymphs of uniform size were selected from a virus-free colony of *A. pisum* and given acquisition access to infected subterranean clover plants for 72 hours. Twenty aphids were then transferred singly onto new sets of healthy test plants every 24 hours until all the aphids had died. The stage of development of each aphid was recorded each day they were transferred.

iv) Acquisition access period (AAP).

Non-viruliferous *A. pisum* were given acquisition access to infected plants for 10, 20, 30, 45 minutes or 1, 1.5, 2, 4, 6, 12, 24, 48 or 72 hours. At the end of the respective acquisition access

periods, the aphids were placed in groups of 10 per plant on healthy test plants until six days after commencement of the experiment (including AAP).

v) Inoculation access period (IAP).

Non-viruliferous *A. pisum* were fed for 72 hours on infected plants and then transferred in groups of 10 to each test plant (ten test plants were used). The aphids were given access to the test plants for either 10, 15, 20, 25, 30 or 45 minutes or 1, 1.5, 2, 4, 6, 12, 24, 48 or 72 hours. The aphids were killed in the fumigation room after completion of each test.

Dependent transmission of viruses by aphids

a) Potyvirus isolates

The results presented in Tables 4.1 and 4.3 indicate that the two BYMV isolates were both transmitted by *R. padi* but failed to infect *T. repens*. By contrast, CYVV-D was not transmitted by *R. padi* and caused a mosaic in *T. repens*.

V. faba cv. Coles Dwarf Prolific plants were mechanically inoculated, either singly or together with CYVV-D and isolates of BYMV. After two weeks, infected leaves of each isolate were placed in Petri dishes. Young nymphs of *R. padi* starved for 3-4 hours at room temperature were allowed to feed on the infected leaves of each isolate for 5 minutes and then transferred to leaves infected with alternate isolates for 5 minutes. The aphids were then placed on healthy *V. faba* test plants for 24 hours (10 aphids per plant, 10 plants per treatment). Each isolate was also inoculated with *R. padi* onto *T. repens* and *V. faba*. *R. padi* were also allowed to feed on the doubly infected leaves for 5 minutes before moving them onto white clover seedlings. These tests were repeated three

times.

b) Luteovirus isolates

As mentioned previously, SDV-As was transmitted specifically by *A. solani* whereas SDV-Ap was transmitted efficiently by *A. pisum* and very inefficiently by *A. solani*. Two host plants that differentiated between the two SDV isolates were *T. repens* (infected by SDV-As only) and *T. pratense* (infected by SDV-Ap only).

A. pisum and *A. solani* were allowed to acquire SDV-Ap and SDV-As respectively from infected subterranean clover leaves for three days and then groups of five *A. solani* and ten *A. pisum* were placed either alone or together on healthy subterranean clover cv. Mt. Barker seedlings (10 plants per treatment). All the aphids were killed after three days of inoculation access period. Five weeks later, the subterranean clover plants displayed leaf reddening typical of SDV infection. The doubly infected plants were cut into two parts, one half being infested with *A. pisum* and the other with *A. solani*. From each half, *A. solani* in groups of five and *A. pisum* in groups of ten were transferred to healthy *T. repens* and *T. pratense* test plants. Results of tests from single infection sources were compared with those from doubly infected plants. DAS-ELISA was used to confirm whether the *T. repens* and *T. pratense* test plants became infected or not.

4.3 Results

Host ranges and symptoms

CYVV isolates generally caused more severe symptoms than BYMV isolates on those host plants that they had in common (Table 4.1). For example, broad bean (*V. faba*) cultivars reacted with severe systemic necrotic spots to CYVV and a systemic mosaic to BYMV. CYVV

TABLE 4.1

Host reactions to inoculation with four potyvirus isolates.

Host plant	Isolates			
	BYMV-F	BYMV-K	CYVV-B	CYVV-D
Apiaceae				
Coriandrum sativum	(+)	(+)	Mo	Mo
Brassicaceae				
Brassica chinensis	-	-	-	-
Brassica napus	-	-	-	-
Capsella bursa-pastoris	-	-	-	-
Chenopodiaceae				
Chenopodium album	CS	CS	CS	CS
Chenopodium amaranticolor	CS	CS	CS	CS
Chenopodium murale	CS	CS	CS,NS	CS,NS
Chenopodium quinoa	CS	CS	SCS	SCS
Spinacea oleracea	CS	CS	CS	CS
Cucurbitaceae				
Cucumis sativus				
cv. Gerkin	-	-	-	-
Crystal apple	-	-	-	-
Fabaceae				
Lathyrus odoratus	Mo	Mo	Mo	Mo
Medicago sativa				
cv. Du Puit	-	-	-	-
Hunter Field	-	-	-	-
Hunter River	-	-	-	-
Spring Field	-	-	-	-
Phaseolus vulgaris				
cv. Dwarf Bountiful	CS	CS	CS,Mo	CS,Mo
Top Crop	CS	CS	CS,Mo	CS,Mo
Redlands Pioneer	CS	CS	CS,Mo	CS,Mo
Pisum sativum				
cv. Greenfeast	-	-	-	-
Onyx	Mo	Mo	Mo	Mo
Scout	Mo	Mo	Mo	Mo
Trifolium incarnatum	Mo	Mo	Mo	Mo
Trifolium repens				
cv. Huia	-	-	Mo	Mo
Pitau	-	-	Mo	Mo
Trifolium pratense				
cv. Montgomery	-	-	-	-

Trifolium subterraneum				
cv. Dwalganup	Mo	Mo	Mo	Mo
Mt. Barker	Mo	Mo	Mo	Mo
Northam First Early	Mo	Mo	Mo	Mo
Pink Flower	Mo	Mo	Mo	Mo
Vicia faba				
cv. Aquadulce Claudia	Mo	Mo	SNS	SNS
Beryl	Mo	-	-	SNS
Coles Dwarf Prolific	Mo	Mo	SNS	SNS
Early Long Pod	Mo	Mo	SNS	SNS
Lamiaceae				
Ocimum basilicum	-	-	-	RS
Solanaceae				
Datura metel	-	-	-	-
Datura stramonium	-	-	-	-
Lycopersicon esculentum				
cv. Whopper	-	-	-	-
Nicotiana clevelandii	(+)	(+)	SCS	SCS
Nicotiana tabacum				
cv. Hickory	-	-	CS,NS	CS
Turkish	-	-	CS,NS	CS
White Burley	-	-	CS	CS
Xanthi	-	-	CS	CS
Physalis floridana	-	-	Mo	Mo

CS = chlorotic spot,
NS = necrotic spot,
S = systemic symptoms,
Mo = mosaic,
RS = ring spot
- = no symptoms, not infected
(+) = symptomless infection as shown by back inoculation or by DAS-ELISA tests

isolates also caused more pronounced symptoms on *C. murale, C. quinoa, N. clevelandii* and *P. vulgaris* than those caused by the BYMV isolates (Table 4.1). The two CYVV isolates were distinguished from BYMV because they systemically infected tobacco (*N. tabacum*) and white clover (*T. repens*). The two isolates of BYMV showed general similarities in host range and symptoms except for *V. faba* cv. Beryl which was susceptible to BYMV-F but not to BYMV-K. Similarly, CYVV-D caused systemic necrosis on *V. faba* cv. Beryl and ring spots symptoms on *Ocimum basilium* whereas CYVV-B did not.

SDV-Ap generally had a more restricted host range than SDV-As and caused milder symptoms on most of its host plants (Table 4.2). Host plant species infected by SDV-Ap or SDV-As showed, in general, stunting, chlorosis, and interveinal yellowing or reddening of older leaves. The leaves were also often thickened and brittle. Symptoms on a number of host plants follow:

Gomphrena globosa

marginal reddening, thickening and downward curling of lower leaves.

Glycine max

dwarfing of plants with shortened petioles and internodes.

Phaseolus vulgaris

most cultivars tested showed interveinal chlorosis, leaf puckering and the leaves were rolled down.

Pisum sativum

the older leaves on several pea cultivars became thickened, brittle, curved downwards and chlorotic.

Trifolium incarnatum

marginal reddening and downward curling of lower leaves.

TABLE 4.2

Host ranges of two soybean dwarf virus isolates.

Host plants	Virus isolates	
	SDV-Ap	SDV-As
Amaranthaceae		
Gomphrena globosa	-	+
Asteraceae		
Sonchus asper	-	-
Brassicaceae		
Brassica napus	-	-
Capsella bursa-pastoris	-	-
Chenopodiaceae		
Beta vulgaris	-	+
Fabaceae		
Arachis hypogaea	-	-
Glycine max		
cv. Tsurunoko	+	+
Lathyrus odoratus	-	-
Lens esculenta	+	+
Medicago hispida	(+)	+
Medicago indica	(+)	+
Medicago sativa		
cv. Hunter Field	-	-
Hunter River	(+)	-
Pioneer RS Kingaray	-	-
Spring Field	-	-
Wairu	-	-
Yates Alfalfa Sprout	-	-
Melilotus indica	-	-
Phaseolus vulgaris		
cv. Pinto III	-	-
Redlands Pioneer	-	-
Red Mexican 37	-	-
Top Crop	-	+

Pisum sativum			
cv.	Champ	-	-
	Charger	+	(+)
	Dot	-	+
	Early Freezer	-	+
	Frisky	(+)	-
	Frosty	(+)	+
	Greenfeast	(+)	(+)
	Ivy	-	-
	Onyx	+	+
	Puget	+	+
	Rally	+	+
	Sybo	+	+
	Trojan	+	+
Trifolium incarnatum		+	+
Trifolium fragiferum		-	-
Trifolium hybridum		-	(+)
Trifolium pratense			
cv.	Montgomery	(+)	-
	Cowgrass	-	-
	Tetraploid	-	-
Trifolium repens			
cv.	Huia	-	(+)
Trifolium subterraneum			
cv.	Bacchus Marsh	+	+
	Dinninup	+	+
	Tallarook	+	+
	Trikkala	+	+
	Woogenellup	+	+
	Yarloop	+	+
Vicia faba			
cv.	Acquadulce Claudia	+	+
	Beryl	+	+
	Coles Dwarf Prolific	+	+
	Compacta	-	+
	Early Long Pod	+	+
	Extra Early Seville	-	+
	Triple White	+	+

\+ = infected by virus isolate
\- = no symptoms, not infected
(+) = symptomless infection as shown by back inoculation or by DAS-ELISA tests

Trifolium subterraneum

the margins of the older leaves showed reddening which generally developed towards the centres of the leaflets.

Vicia faba

most cultivars showed interveinal yellowing, thickening and upward rolling of the lower and intermediate leaves.

SDV-Ap did not infect white clover (*T. repens*), the most important reservoir of SDV-As infection in Tasmania. However some cultivars of lucerne (*M. sativa*), red clover (*T. pratense*) and pea (*P. sativum*) were infected by SDV-Ap but not SDV-As (Table 4.2).

Aphid transmission

a) Potyvirus isolates

There were many interactions in the efficiencies of transmission between the nine aphid species and the four virus isolates (Tables 4.3, 4.4). In general, the BYMV isolates were transmitted more readily than the CYVV isolates but there were exceptions to this rule. For example *A. solani* transmitted BYMV-F very poorly compared to the two CYVV isolates. The CYVV-B isolate was more readily transmitted than CYVV-D by almost all of the aphid species tested. The efficiencies with which the two BYMV isolates were transmitted by individual species were often at variance, *R. padi* for example transmitting the F isolate more efficiently while *A. solani* and *M. euphorbiae* were more effective in transmitting BYMV-K. The six aphid species that are known to colonise broad bean (*A. kondoi, A. pisum, A. craccivora, A. solani, M. euphorbiae* and *M. persicae*) were generally more efficient vectors of all four isolates when compared to the three non-colonising species. *Hyperomyzus lactucae* failed to transmit even though it probed on the virus

TABLE 4.3

Transmission (%) of four legume potyvirus isolates by nine aphid species.

Aphid species	Virus isolate			
	BYMV-F	BYMV-K	CYVV-B	CYVV-D
Acyrthosiphon kondoi	56	61	25	19
Acyrthosiphon pisum	77	67	40	40
Aphis craccivora	80	73	70	50
Aulacorthum solani	25	90	80	73
Cavariella aegopodii	7	10	8	7
Hyperomyzus lactucae	0	0	0	0
Macrosiphum euphorbiae	67	86	55	40
Myzus persicae	83	73	50	33
Rhopalosiphum padi	43	23	10	0

LSD's at 5, 1 and 0.1% between species within isolates and between isolates within species (excluding data for *H. lactucae*) = 22, 29, 37.

TABLE 4.4

Probabilities of single aphids transmitting four potyvirus isolates.

Aphid species	Virus isolate			
	BYMV-F	BYMV-K	CYVV-B	CYVV-D
Acyrthosiphon kondoi	0.08	0.09	0.03	0.02
Acyrthosiphon pisum	0.14	0.10	0.05	0.05
Aphis craccivora	0.15	0.12	0.11	0.07
Aulacorthum solani	0.03	0.21	0.15	0.12
Cavariella aegopodii	0.01	0.01	0.01	0.01
Hyperomyzus lactucae	0.00	0.00	0.00	0.00
Macrosiphum euphorbiae	0.10	0.18	0.07	0.05
Myzus persicae	0.16	0.12	0.07	0.04
Rhopalosiphum padi	0.05	0.03	0.01	0.00

sources and test plants, while *Cavariella aegopodii* was an inefficient vector of all four isolates. However the non-colonising *R. padi* was moderately efficient in its transmission of the BYMV isolates and this fact was of importance in the field from an epidemiological viewpoint (Chapter 6). *R. padi* transmitted CYVV-B poorly and CYVV-D not at all.

b) Soybean dwarf virus isolates

A. solani was the only aphid species which transmitted the SDV-As isolate (Table 4.5). It was a very efficient vector of this isolate. Similarly, the SDV-Ap isolate was transmitted very efficiently by *A. pisum* (93%). It was also transmitted inefficiently by *A. solani* (13%) and *M. euphorbiae* (5%). *A. kondoi, A. craccivora* and *M. persicae* failed to transmit either SDV-Ap or SDV-As.

SDV-Ap was transmitted by young nymphs of *A. pisum* more efficiently than by adult apterae (Table 4.6). Adult alatae were poor vectors of SDV-Ap. The rate of transmission increased when larger numbers of aphids were transferred from virus sources to test plants. One hundred per cent of transmission was obtained when ten nymphs were allowed to feed on each test plant.

In tests to examine the persistence of transmission of SDV-Ap by *A. pisum*, 13 of 20 aphids acquired virus and infected test plants (Table 4.7). Several aphids retained their infectivity after moulting and three did so after developing into adult alatae. In the serial transfer tests, individual aphids varied considerably in their abilities to transmit the virus. Transmission usually occurred intermittently. *A. pisum* was highly efficient in virus transmission during the first ten days after the commencement of the acquisition access period and then largely lost its transmitting

TABLE 4.5

Aphid transmission of two isolates of SDV to *Trifolium subterraneum* cv. Mt. Barker.

Aphid species	SDV isolates	
	SDV-Ap	SDV-As
Acrythosiphon kondoi	0/20*	0/20
Acyrthosiphon pisum	92/99	0/98
Aphis craccivora	0/20	0/20
Aulacorthum solani	13/98	95/100
Macrosiphum euphorbiae	1/20	0/20
Myzus persicae	0/20	0/20

* Number of infected plants/number of plants inoculated

TABLE 4.6

Transmission (%) of SDV-Ap by different morphs and numbers of *A. pisum*.

Morph	Number of aphids/plant		
	1	5	10
young nymphs	63	80	100
adult apterae	15	34	50
adult alatae	5	20	35

TABLE 4.7

Serial transmission of SDV-Ap by single *A. pisum* given a 72 hour acquisition access period on an infected subterranean clover plant.

Aphid No.	Serial transfers to healthy subterranean clover test plants					
	1[(a)]	10	20	30	40	50
1	----------*------d					
2	-+---+-----*-----+--------------d					
3	-+++---d					
4	----------*--------------------------------d					
5	----+-----d					
6	---+-+++----*------d					
7	----------*----------------------d					
8	---+++-+-+-*-------d					
9	-------------*-------------d					
10	--------------*-------------d					
11	--+-+--------*---------------------d					
12	--+-+---------*d					
13	-+++++-++---*----d					
14	--+-+-++-+---+*+--------------------------d					
15	+----+---+----*-+-------d					
16	----------*----------------------d					
17	-+---+---+---*----------------------------d					
18	-+--+--+-+--*---d					
19	------------*---------d					
20	-+--+-+------*--------------d					

(a) Days after leaving source plant
+ indicates infection
- no infection
d death
* alate

ability during subsequent transfer periods (Figure 4.1).

No transmission of SDV-Ap was recorded when the AAP was less than 1.5 hours (Figure 4.2). Increasing the AAP beyond this time increased the efficiency of transmission and the optimal AAP was at least 48-72 hours. Similarly, *A. pisum* was able to transmit SDV-Ap within 20 minutes after becoming infective. The rate of transmission increased as the IAP was extended up to 24 hours. The aphids transmitted the SDV-Ap isolate with 100% efficiency when the IAP was from 24-72 hours.

No tests were done to obtain information on the length of any (probable) incubation period of the virus in the aphids.

Dependent transmission of plant viruses by aphids

a) Potyvirus isolates

The HC encoded by BYMV-F was able to assist *R. padi* to transmit CYVV-D (Table 4.8). This occurred whether the *R. padi* were given access to a source of BYMV-F before feeding on a CYVV-D or whether they were allowed to feed on plants infected with both viruses together. No transmission of CYVV-D by *R. padi* occurred if the aphids were given access to CYVV-D before access to the BYMV-F isolate. The BYMV-K HC did not assist the transmission of CYVV-D by *R. padi* in the limited numbers of tests undertaken to check this point .

Several attempts were made to determine whether HC present in plants infected with BYMV-K could assist in improving the efficiency of transmission of BYMV-F by *A. solani* (see Table 4.3). A variety of methods were used to prepare HC and virus separately from plants infected with BYMV-F and with BYMV-K based on those reported by Govier *et al.*(1977), Pirone (1981) and Sako and Ogata (1981a). *A. solani* were allowed to feed through stretched Parafilm membranes on

TABLE 4.8

Effect of BYMV-F and BYMV-K induced helper components on the transmission of CYVV-D by *R. padi*.

Feeding source	Transmission rate		
	Experiment		
	1	2	3
(a)			
BYMV-F, then CYVV-D	3/10	2/10	2/9
BYMV-K, then CYVV-D	0/10	0/10	0/10
CYVV-D, then BYMV-K	0/9	0/10	0/10
CYVV-D, then BYMV-K	0/10	0/10	0/10
(b)			
BYMV-F + CYVV-D	2/10	1/10	1/10
BYMV-K + CYVV-D	0/10	0/10	0/10
(c)			
BYMV-F, then *Trifolium repens*	0/10	0/9	0/8
Vicia faba	5/10	4/10	5/9
BYMV-K, then *T. repens*	0/10	0/10	0/9
V. faba	2/10	2/9	0/10
CYVV-D, then *T. repens*	0/10	0/9	0/10
V. faba	0/10	0/10	0/10

(a) *R. padi* fed on the first virus source for 5 minutes before being transferred to feed on the second virus source for 5 minutes and then moved to feed on healthy white clover cv. Huia for 24 hours (10 aphids per plant ; potyviruses maintained on broad bean).

(b) *R. padi* fed on doubly infected plants for 5 minutes and then transferred to healthy white clover cv. Huia for 24 hours (10 aphids per plant).

(c) *R. padi* fed on the virus sources for 5 minutes and then transferred to feed on *Trifolium repens* or *Vicia faba* (10 aphids per plant).

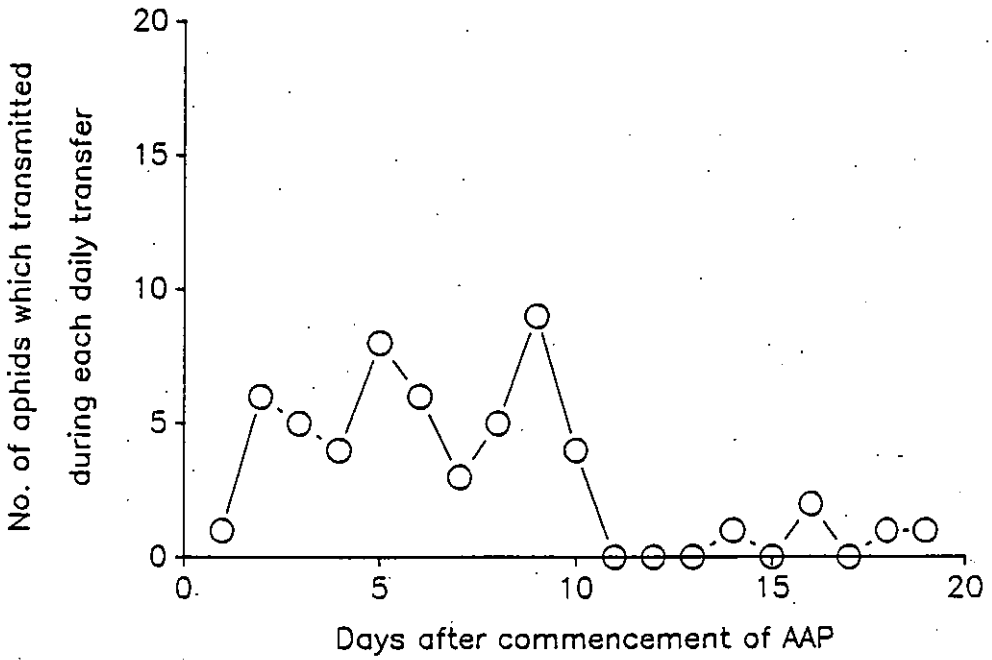


FIGURE 4.1

Number of transmissions of SDV-Ap by 20 single *A. pisum* during daily transfers.

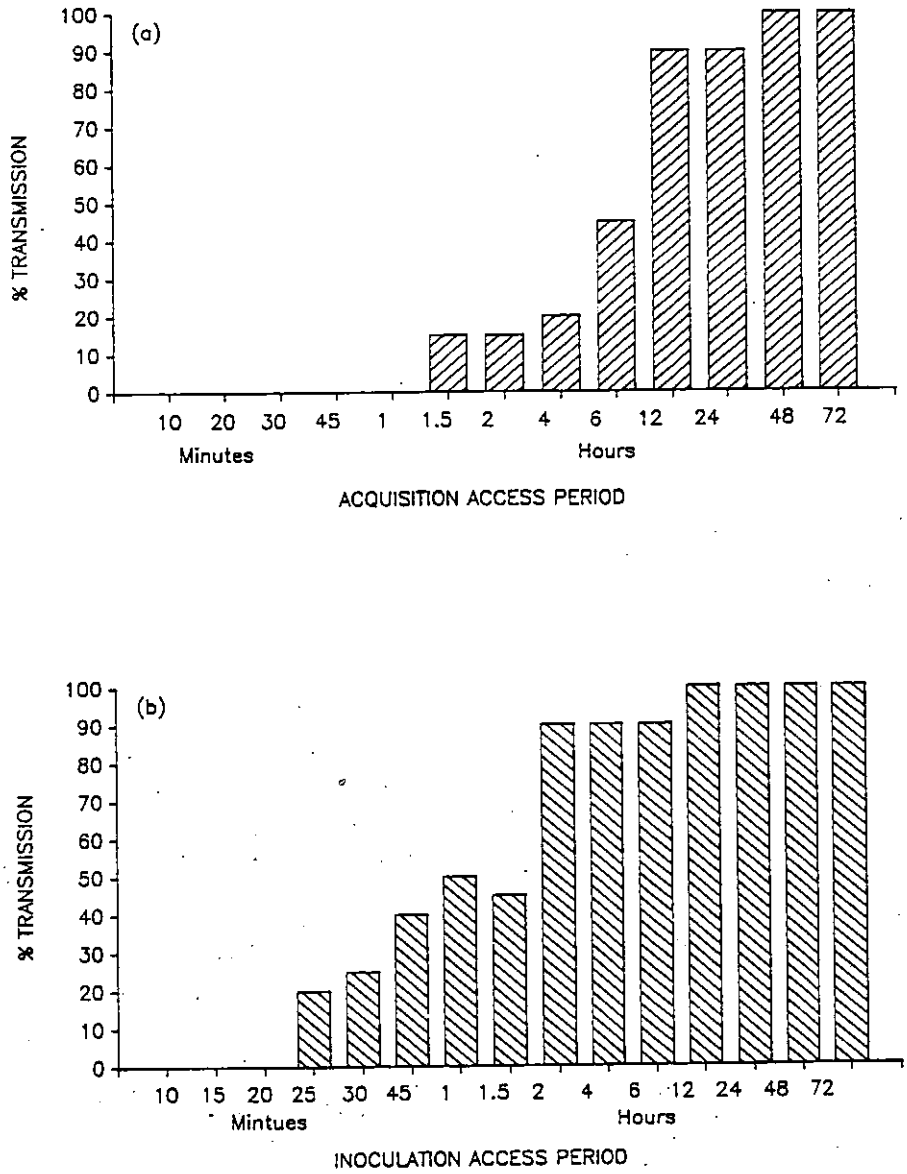


FIGURE 4.2

Effect of different acquisition access (a) and inoculation access (b) periods on the transmission of SDV-Ap by *A. pisum*.

all permutations and combinations of healthy sap extracts, infected sap extracts, purified virus preparations (F and K adjusted to 100μg/ml) and HC preparations, all made up to 20% with sucrose. However every attempted transmission failed, in six separate experiments involving various modifications of preparation of HC's from the plants infected with each isolate. It was therefore not possible to prove that HC from plants infected with BYMV-K could improve the efficiency of BYMV-F by *A. solani* and thus ratify earlier reports on the specificity of interactions between HC's potyviruses and their aphid vectors (Kassanis, 1961 ; Sako & Ogata, 1981a ; Lecoq & Pitrat, 1985). These failures to transmit the BYMV isolates from solutions through Parafilm membranes contrasted with the efficient transmission of SDV-Ap from purified preparations by *A. pisum* in these investigations, and of SDV-As by *A. solani* (Johnstone et al., 1982).

No attempt was made to establish whether HC purified from plants infected with BYMV-F could assist in dependent transmission of CYVV-D by *R. padi*.

b) Luteovirus isolates

SDV-Ap and SDV-As were both transmitted in a dependent manner from mixed infections by *A. solani* and *A. pisum* respectively (Table 4.9). Although *A. solani* occasionally transmitted SDV-Ap from plants infected with this virus alone, it regularly transmitted SDV-Ap from plants infected with both isolates in all experiments (40%). From the same doubly infected plants, *A. pisum* transmitted SDV-As in all the experiments, but the rate of transmission was lower (26.4%). Therefore, *A. solani* was a more effective helper virus in the dependent transmission of SDV-Ap than *A. pisum* was as

TABLE 4.9

Results of tests for dependent virus transmission from mixed infections of the SDV-Ap and SDV-As isolates of soybean dwarf virus.

Virus tested in role shown		Aphid vector	Number of plants from which the dependent virus was transmitted over number tested for each kind of infection*	
Dependent	Helper		Single[(a)]	Double[(b)]
SDV-Ap	SDV-As	*A. solani*	0/10	2/9
			1/10	3/9
			2/18	9/20
			1/20	9/17
			2/15	5/15
SDV-As	SDV-Ap	*A. pisum*	0/10	1/8
			0/10	3/10
			0/20	7/20
			0/18	5/19
			0/15	3/15

* Virus identification using DAS-ELISA

(a) Subterranean clover cv. Mt. Barker seedlings were used as indicator plants to test for transmission of the dependent virus alone.

(b) *Trifolium pratense* cv. Montgomery seedlings were used as indicator plants for the dependent transmission of SDV-Ap by *A. solani* and *Trifolium repens* cv. Huia for the dependent transmission of SDV-As by *A. pisum*. *T. pratense* was found to be immune to SDV-As and *T. repens* immune to SDV-Ap.

a helper virus for SDV-As (in the presence of SDV-Ap).

4.4 Discussion

Bean yellow mosaic virus, clover yellow vein virus and pea mosaic virus (PMV) were classified as members of the BYMV subgroup of potyviruses, on the basis of their host ranges and symptomatology (Bos *et al.*, 1974, 1977 ; Jones & Diachun, 1977) and their serological specificities (Moghal & Franki, 1976a ; Barnett *et al.*, 1987). Host ranges have been widely used for identifying potyviruses although the severity of symptoms is dependent upon environmental conditions (Bos *et al.*, 1974). The results from host range tests (Table 4.1) indicated that the two isolates of BYMV (BYMV-F and BYMV-K) and the two isolates of CYVV (CYVV-B and CYVV-D) could be clearly distinguished as two separate groups of viruses. CYVV caused more severe symptoms on tobacco (*N. clevlandii*), common bean (*P. vulgaris*), broad bean (*V. faba*) and *C. quinoa* than those caused by BYMV. This confirmed earlier reports by Munro (1981), Forster & Musgrave (1985) and Barnett *et al.* (1987). In addition, CYVV infected white clover (*T. repens*) whereas red clover (*T. pratense*) tends to be naturally infected by some isolates of BYMV (Barnett & Gibson, 1975 ; Jones & Diachun, 1976 ; McLaughlin, 1983).

The efficiency of transmission of viruses with aphid vectors depends on many factors including aphid biotype, aphid behaviour, virus strains, host plants and environmental conditions (Thottappilly *et al.*, 1972). Many aphid species have been reported as vectors of BYMV and CYVV including *A. pisum, A. solani, M. euphorbiae* and *M. persicae* (Bos, 1970 ; Hollings & Stone, 1974). In the work reported here, eight of the nine aphid species tested transmitted BYMV and CYVV (Table 4.3, 4.4). However there were very

significant interactions in efficiencies of transmission between the virus isolates and aphid species.

At Cambridge, in south-eastern Tasmania, *A. pisum* and *A. craccivora* were found most commonly in the experimental broad bean plots and these were efficient vectors of the BYMV and CYVV isolates. However apterae of *A. craccivora* in particular, and *A. pisum* to a lesser extent, do not move readily between plants within crops. The source of *A. pisum* was presumed to be stands of lucerne growing nearby whereas *A. craccivora* apparently migrates in wind currents from mainland Australia (Johnstone, 1983a). *R. padi* may be important as a vector of BYMV in bean and pea crops if cereal crops are growing nearby.

In north-western Tasmania, *A. solani* and *M. persicae* are more common and seem more important as vectors of SDV and potyviruses respectively as important host plants of these two aphid species (white clover and potato) are common in this region.

SDV-As has many similarities to SDV isolates described from Japan (yellowing strain, SDV-Y) and New Zealand (SDV-NZ) (Tamada, 1970 ; Wilson & Close, 1973). They were transmitted principally by *A. solani*, occurring commonly in white clover (*T. repens*) and common bean (*P. vulgaris*) in those countries (Tamada, 1970 ; Ashby *et al.*, 1979 ; Johnstone *et al.*, 1984a). In contrast, SDV-Ap appears to be related to legume yellows luteovirus (LYV) (Duffus, 1979) and SDV isolates from California (Johnstone *et al.*, 1984b ; Johnstone, Liu & Duffus, unpublished data) on the basis of its host range (legumes only) and aphid vector specificities. These isolates were all transmitted efficiently by *A. pisum* and inefficiently by *A. solani*. They also infected lucerne (*M. sativa*) an important crop in the areas where SDV-Ap infections were commonly found (see Chapter 6).

It seems that there has been a selection in areas where *Trifolium* spp and *A. solani* are common for SDV types transmitted specifically by *A. solani* (in north-western Tasmania) and a selection for *A. pisum* specific types in areas (south-eastern Tasmania ; Gilroy and Salinas in California) where *Medicago* spp and pea aphids predominate (Johnstone, personal communication). The differences in host ranges of SDV-Ap and SDV-As seemed due to differences in the isolates rather than differences in the host ranges of their aphid vectors.

SDV-Ap and SDV-DS (dwarfing strain from Japan) had similar host ranges but differed in aphid specificity (Tamada, 1973 ; Tamada & Kojima, 1977). These two isolates had host ranges restricted to legumes and generally caused milder symtoms than SDV-As and SDV-Y.

The mode of aphid transmission of SDV-Ap is quite similar to that of other members of the luteovirus group, characterised by a well marked vector specificity, retention of infectivity after moulting and persistence of infectivity for more than one week before declining (Waterhouse *et al.*, 1988). Different morphs of the prime aphid vector affected the efficiency of virus transmission. Young nymphs were more effective in transmission than adult apterae or adult alatae. Many suggestions based on physiological differences between nymphs and adult aphids have been put forward to explain this, such as accumulation of virus in salivary glands of nymphs being more rapid than in adults (Zhou & Rochow, 1984). In addition, young nymphs are less mobile than adults and thus more likely to spend their time feeding (Damsteegt & Hewings, 1986).

Dependent transmission of viruses from mixed infections is a feature of many plant viruses transmitted by aphids. Dependent transmission of non-persistent viruses can occur not only when

aphids feed on doubly-infected plants, but also when they first feed on a source of the helper virus before acquiring the dependent viruses (Table 4.8). This feature suggested that the HC might act by allowing the dependent virus to become bound to the aphids' stylets from which it can later be released (Govier & Kassanis, 1974b). BYMV-K did not assist in the dependent transmission of CYVV-D by *R. padi* presumably because it did not code for the production of a HC that integrated the surface features of itself, its HC and the aphids' stylets. Another reason for the success of the BYMV-F HC may have been that it prevented the aggregation and precipitation of virus particles in imbibed sap (Govier & Kassanis, 1974b; Lopez *et al.*, 1981).

By contrast, because of the need for interactions between the dependent and helper viruses in the plants, dependent transmission of luteoviruses only occurred when the aphid vectors fed on doubly-infected plants. The mechanism of dependent transmission of luteoviruses appears to involve heterologous encapsidation which describes a phenomenon whereby the nucleic acid of the dependent virus becomes at least partially encapsidated within helper virus protein capsid (Rochow, 1977). Dependent transmission by aphids is known to occur among five serotypes of barley yellow dwarf luteovirus. Degrees of efficiency in dependent transmission varied with different combinations of vector species and serotype. The SDV-As isolate was more effective in enabling *A. solani* to transmit SDV-Ap than the SDV-Ap isolate was in enabling *A. pisum* to transmit SDV-As. Dependent transmission phenomena have been helpful in understanding not only interactions between helper and dependent viruses but also in explaining various aspects of vector specificity and the epidemiology of plant viruses in the field (Chapter 1).

CHAPTER 5

Physical properties of selected potyviruses and luteoviruses.

5.1 Introduction

The physical properties of plant viruses including properties of purified virus preparations and their serological reactivities have often been particularly useful for identifying virus strains or groups and for their routine detection in plant samples.

Different procedures have been reported for the purification of BYMV and CYVV isolates, varying with respect to propagative host plants, extraction buffers, clarifying agents and resuspending buffers (Hollings & Brunt, 1981). Some strains of BYMV and CYVV are easily purified and high yields (up to 20mg/kg leaf tissues) obtained. However, there are often major problems involved in preparing purified potyvirus preparations due to such factors as the irreversible aggregation of virus particles during or following extraction, and instability and particle breakage following treatment with some chemicals (Bos, 1970 ; Hollings & Stone, 1977).

The particles of SDV, like those of other members of the luteovirus group, are confined to phloem tissues (Jayasena *et al.*, 1981). Very low yields of virus particles were obtained from infected plants by conventional methods, presumably because of the difficulty in disrupting the phloem cells sufficiently to release the particles (Kojima & Tamada, 1976). However, Paliwal (1978) demonstrated that incubating homogenates of plants infected with barley yellow dwarf virus with cellulase increased the yield of virus by 20%. Furthermore, Takanami and Kubo (1979) showed that a

commercial pectinase and cellulase mixture, Driselase^{R}, gave good yields of virus particles when used to purify potato leafroll and tobacco necrotic dwarf viruses. Driselase^{R} and Cellulase type I (Sigma) have been used in the purification of luteoviruses (Tamada & Harrison, 1980 ; Waterhouse & Murant, 1981 ; Ashby & Kyriakou, 1982). Celluclast^{R}, an industrial grade cellulase, has been used recently for extraction because its cost was lower than laboratory-grade cellulase (Johnstone *et al.*, 1982 ; Waterhouse & Helms, 1984 ; Hewings *et al.*, 1986). The three critical steps in the purification and concentration of luteoviruses are the initial propagation of virus in plants in the glasshouse, the extraction procedure and the final stages of purification (Johnstone, 1983a). Virus concentration increased in plants grown under moderately cool conditions (Helms *et al.*, 1983) and virus yields were therefore greater during winter (Ashby & Kyriakou, 1982 ; D'Arcy *et al.*, 1983). Extraction procedures for the efficient release of the luteovirus particles from the phloem elements have also included pulverizing the tissues while they were frozen, ultra-high speed homogenizing and use of enzyme preparations (Johnstone *et al.*, 1982 ; Hewings *et al.*, 1986). The concentrated virus should be passed through a sucrose density gradient fractionator at least twice to completely separate the virions from the normal host constituents (Johnstone, 1983a).

Serology is a most useful tool for the identification or classification of viruses and for the diagnosis of plant infections. Methods used include precipitin tests, immunodiffusion tests, various labelled antibody techniques and immunoelectron microscopy (Van Regenmortel, 1982). Enzyme-labelled antibodies were first used for the detection of virus antigens in tissue sections in 1966

(Wicker & Avrameas, 1969). Their use in quantitative procedures was reported by Engvall and Perlmann (1971). The method known as enzyme-linked immunosorbent assay (ELISA) was used for the diagnosis of virus diseases in polystyrene micro-titre plates and for the measurement of very low concentrations of virus and specific antibody (Voller *et al.*, 1976).

The visualization of immunological reactions on electron microscope grids is one the most sensitive serological techniques (Derrick, 1973 ; Van Regenmortel, 1978). Three different approaches widely used in immune electron microscopy (IEM) [(reviewed by Milne and Luisoni, 1977)] are clumping or decoration, antibody coating, and "trapping" on antibody-coated grids. The benefits of IEM are speedy results where small numbers of samples are involved, sensitive detection of viral relationships, economy of antiserum usage, and high specificity.

The aims of the studies reported here were to develop procedures for the purification of BYMV-F, CYVV-D and SDV-Ap, produce polyclonal antisera to them in rabbits, and to use those antisera to investigate the relationships between the potyvirus isolates and luteovirus isolates using various serological techniques.

5.2 Materials and Methods

Virus sources and propagations

BYMV-F was propagated in pea (*Pisum sativum*) cv. Onyx, and CYVV-D in tobacco (*Nicotiana clevelandii*) following mechanical inoculation, and SDV-Ap in *P. sativum* cv. Trojan following aphid inoculation. Infected tissue of *P. sativum* cv. Onyx and *N. clevelandii* grown from seeds in sterilized soil was harvested 14-18

days after inoculation and cooled at 4° for 1-2 hours before commencing extraction. The *P. sativum* cv. Trojan plants were grown from seed in vermiculite in plastic pots and watered with Hoagland's nutrient solution (Hewitt, 1966) at least twice a week. The roots and shoots of infected plants were harvested 30-40 days after inoculation with SDV-Ap and frozen at -20° pending viral purification. Earlier tests had indicated that virus levels were higher in cv. Trojan than in the other pea cultivars listed in Table 4.2.

Purification procedures

The three virus isolates were centrifuged using a high speed centrifuge (MSE High Speed 18) and an ultracentrifuge (Beckman L2-65). Sucrose density gradient columns for further purification of virus preparations were prepared in 80mm x 27mm cellulose nitrate tubes by layering 40% (7ml), 30% (7ml), 20% (7ml) and 10% (4ml) sucrose, all in resuspending buffer. The purified viruses were finally stained with 2% uranyl acetate and viewed with an electron microscope to measure their dimensions. A spectrophotometer was used to estimate virus yields assuming the extinction coefficients to be 2.4 and 7.3 $(mg/ml)cm^{-1}$ for potyviruses and luteoviruses respectively at 260nm after correcting for light scattering (Moghal & Francki, 1976a ; Ashby & Kyriakou, 1982). The different buffers and reagents finally selected for purification of the three virus isolates, after extensive testing are listed in Table 5.1.

The purification procedures for BYMV-F were modified from the methods of Jones and Diachun (1976, 1977). Infected tissue of *P. sativum* cv. Onyx was homogenized in 0.5 M potassium phosphate (pH 7.0) containing 1 M urea, 0.5% thioglycolic acid (TGA) and 0.01 M

TABLE 5.1

Buffers and chemical additives used for the purification of BYMV-F, CYVV-D and SDV- Ap.

	BYMV-F	CYVV-D	SDV-Ap
Extracting buffer	0.5M phosphate buffer, pH 7.0	0.5M borate buffer, pH 7.8	0.1M citrate buffer, pH 6.0
Additives	1M urea, 0.5% TGA, 0.01M DIECA	0.01M $MgCl_2$, 0.2% 2-ME	0.02% NaN_3, 0.1% TGA
Enzymatic digestion	-	-	1% celluclast
Clarification	chloroform + carbon tetrachloride	chloroform	butanol + chloroform
Resuspending buffer	0.5M phosphate buffer, pH 7.0 (+1M urea, 0.1% 2-ME)	0.05M borate buffer, pH 8.0 (+1M urea + 0.1% 2-ME)	0.1M phosphate buffer, pH 7.0, (+0.01M glycine)

DIECA = sodium diethyldithiocarbamate
$MgCl_2$ = magnesium chloride
2-ME^2 = 2-mercaptoethanol
NaN_3 = sodium azide
TGA^3 = thioglycolic acid

sodium diethyldithiocarbamate (DIECA) at a ratio 1:1.5 (w:v). The extract was clarified for 1-2 minutes with one half-volume (v/v) each of chloroform and carbon tetrachloride, and the emulsion was broken by centrifugation at 10,000*g* for 10 minutes. Polyethylene glycol (PEG, MW 6000) and sodium chloride (NaCl) were added to final concentrations of 4% and 0.2 M respectively and the mixture was then stirred for 30 minutes before leaving to stand at 4° for 1 hour. Precipitated virus was recovered by centrifugation at 10,000*g* for 15 minutes and then resuspended by gentle agitation for 2 hours at 4° in one-fifth the original volume of 0.5 M phosphate buffer pH 7.0 containing 0.1% 2-mercaptoethanol (2-ME) and 1 M urea (resuspending buffer) (Uyeda *et al.*, 1975 ; Vovlas & Russo, 1978). The extracts were clarified by centrifugation at 4000*g* for 15 minutes and the supernatants then centrifuged for 90 minutes at 75,000*g* (Beckman 30 rotor) in tubes with 4 ml of 30% sucrose cushions in buffer (Reddick & Barnett, 1983). The pellets were again resuspended in 0.5 M phosphate buffer by standing overnight at 4°. The differential centrifugation step was repeated once and the viruses were then further purified by rate-zonal centrifugation in 10-40% sucrose density gradient columns (in 0.5% M phosphate buffer + 1 M urea, pH 7.0) at 60,000*g* for 2.5 hours (Beckman SW 25.1 rotor). Centrifuged gradient columns were scanned at 254nm using an ISCO UA5 density gradient fractionator. The fractions containing virus were centrifuged at 75,000*g* for 2.5 hours (Beckman 30 rotor). Pellets were finally resuspended in phosphate buffer saline (PBS = 0.01 M sodium phosphate buffer containing 0.85% NaCl, pH 7.0). The purified virus preparations were further examined by electron microscopy prior to use for production of polyclonal antisera.

The purification procedure for CYVV-D was modified from the

method of Brunt and Kenten (1971). *N. clevelandii* leaves infected with CYVV-D were homogenized in a Waring blender with two volumes (w/v) of 0.5 M borate buffer pH 7.8 containing 0.01 M magnesium chloride ($MgCl_2$) and 0.2% 2-ME. The crude sap was added to one-half volume of chloroform and the mixture homogenized for 1-2 minutes. The emulsion was broken by centrifugation at 10,000*g* for 15 minutes and the aqueous phase filtered through glass wool. PEG (MW 6000) was added to a final concentration of 4% and the mixture then gently stirred for 30 minutes before leaving it to stand at 4° for one hour. Precipitated virus was recovered by centrifugation at 10,000*g* for 15 minutes and resuspended in one-tenth the original volume of extracting buffer by standing at 4° for 24 hours. Extracting buffer was then added back to the original volume of the sap extract before concentrating the virus by two cycles of differential centrifugation (10 minutes at 10,000*g* then 90 minutes at 76,000*g* through a 4ml cushion of 30% sucrose using a Beckman 30 rotor). The pellets were resuspended by stirring in 0.05 M borate buffer pH 8.0 containing 1 M urea and 0.1% 2-ME for one hour. The suspensions were then clarified by centrifuging at 6000*g* for 10 minutes before layering on linear gradients of 10-40% sucrose buffered in 0.05 M borate buffer pH 8.0 (with 1 M urea) and centrifuging in a Beckman SW 25.1 rotor at 60,000*g* for 2.5 hours. Gradients were fractionated with the ISCO density gradient fractionator. The virus-containing fractions were centrifuged at 75,000*g* for 2.5 hours. The pellets were resuspended in 0.05 borate buffer pH 8.0 and were then examined by electron microscopy prior to use for polycolonal antiserum production as before.

The purification procedure for SDV-Ap was modified from the method described by Johnstone *et al.* (1982). Infected tissue of *P.*

sativum cv. Trojan (including roots) was extracted in 0.1 M citrate buffer pH 6.0 (2ml/g leaf) containing 0.1% TGA, 0.02% sodium azide and 1% Celluclast (an industrial grade of cellulase prepared from *Trichoderma viride*, Calbiochem). After shaking the homogenate at room temperature for 16 hours, the slurry was homogenized in a VirTis blender and squeezed through cheesecloth. One-fourth volume of a 1:1 mixture of chloroform and n-butanol was added and the mixture homogenized for 2-3 minutes. The emulsion was broken by centrifugation at 10,000*g* for 15 minutes at 20°. PEG - 6000 and NaCl were added to 6% and 0.2 M respectively and the solution stirred for one hour, left at room temperature for one hour, then centrifuged at 10,000*g* for 20 minutes at 20°. The pellets were resuspended in 0.1 M potassium phosphate pH7.0 containing 0.01 M glycine (PBG) by standing overnight at 4°. The suspension was further purified and concentrated by two cycles of differential centrifugation (8000*g* for 15 minutes and either at 76,000*g* for 4 hours in a Beckman 30 rotor or 100,000*g* for 3 hours in a Beckman 40 rotor). Samples of partially purified virus were layered onto sucrose density gradients prepared in PBG buffer and then centrifuged for 3.5 hours at 60,000*g* in a Beckman 25.1 rotor at 20°. The gradients were scanned and the virus containing fractions collected using an ISCO UA-5 density gradient fractionator. The virus was recovered by centrifuging at 100,000*g* for four hours (Beckman 40 rotor). The purified preparations were adjusted to 20% with sucrose and then their infectivities were tested by feeding *A. pisum* on them through Parafilm^{R} membranes. Other aliquots of purified virus were examined in the electron microscope prior to use for polyclonal antiserum production.

Antisera production

Antisera to BYMV-F, CYVV-D and SDV-Ap were produced in rabbits using 3 intramuscular injections followed by an intravenous injection, all at 14 day intervals. For intramuscular injections, purified virus preparations were emulsified with an equal volume of Freund's incomplete adjuvant. Each injection consisted 500-600, 300-400 and 200-250 µg of purified BYMV-F, CYVV-D and SDV-Ap respectively. The rabbits were bled at weekly intervals from two weeks after the final injection. Sera separated from the blood were made to 0.02% with sodium azide or 1:1 (v/v) with glycerol and stored at -20°.

Serological tests

The methods for DAS-ELISA tests were based on those described by Clark and Adams (1977). The IgG fractions of the antisera to BYMV-F, CYVV-D and SDV-Ap were purified by ammonium sulphate precipitation and DEAE (Whatman DE 22 diethylaminoethyl) cellulose chromatography and then conjugated with alkaline phosphatase as described in Chapter 2.6. Optimal concentration of coating IgG and dilution of conjugated IgG were done using DAS-ELISA and volumes of 200µl per well. Ninety six wells of a polystyrene microtitre plate were coated with 0.37, 1.11 and 3.0µg/ml of purified IgG in carbonate coating buffer (pH 9.6). The plate was incubated overnight at 4° and rinsed. Virus infected and uninfected tissues were ground in phosphate buffer ph 7.4 containing 2% polyvinylpyrolidone (MW 40,000) and 0.2% bovine serum albumin (extracting buffer) and centrifuged for 3 minutes at 5000*g*. The infected tissue extract, uninfected tissue extract and extracting buffer were added to 48, 36 and 12 wells respectively. The

dilutions of tissue extracts tested were of 1:3.7, 1:11.1, 1:33.3 and 1:100 while the dilutions of healthy tissue extract were 1:3.7, 1:11.1 and 1:33.3. The plate was incubated at 37° for 4 hours. After rinsing with PBS-Tween, conjugated IgG was diluted in the extracting buffer and added to 96 wells at dilutions of 1:250, 1:750, 1:2250 and 1:6750. The plate was incubated at 4° overnight and then rinsed with PBS-Tween. The final step was to add substrate (0.6mg/ml *p*-nitrophenyl phosphate) and the reactions were read using Titertek Multiskan^{R} photometer at 405nm.

The relative concentrations of antibodies to BYMV-F, CYVV-D and SDV-Ap in the different bleeds were estimated using indirect ELISA tests. Polystyrene microtitre plates were coated with infected leaves (1:5, w/v) extracted in carbonate coating buffer (pH 9.6) for 4 hours at 37°. The plates were rinsed with PBS-Tween three times and the weekly serum samples containing antibodies to BYMV-F, CYVV-D and SDV-Ap were incubated at their optimal dilutions (1:2000, 1:2000 and 1:1000 respectively) for 24 hours at 4°. After rinsing with PBS-Tween, the plate was incubated with a sheep anti-rabbit enzyme conjugate (1:3000, v/v) for four hours at 37° and then rinsed. Finally substrate (*p*-nitrophenyl phosphate 0.6mg/ml) was added.

The two isolates of BYMV and CYVV were compared serologically following the method previously described in Chapter 2.6, using polyclonal antibodies (BYMV-F and CYVV-D) in DAS-ELISA tests and by indirect ELISA using a monoclonal antibody (DS 6-Fusion 1-16-B from Dr. D. D. Shukla, CSIRO Division of Biotechnology, Melbourne). The dilutions of monoclonal antibody in extracting buffer were undiluted, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. Immune electron microscopy was also used to examine and identify these potyvirus

isolates by both trapping and decoration using the antisera produced to BYMV-F and CYVV-D and antisera to two defined isolates each of BYMV (#226,307) and CYVV (#87,94) kindly provided by Dr. A. A. Brunt, Glasshouse Crops Research Institute, Littlehampton, U K.

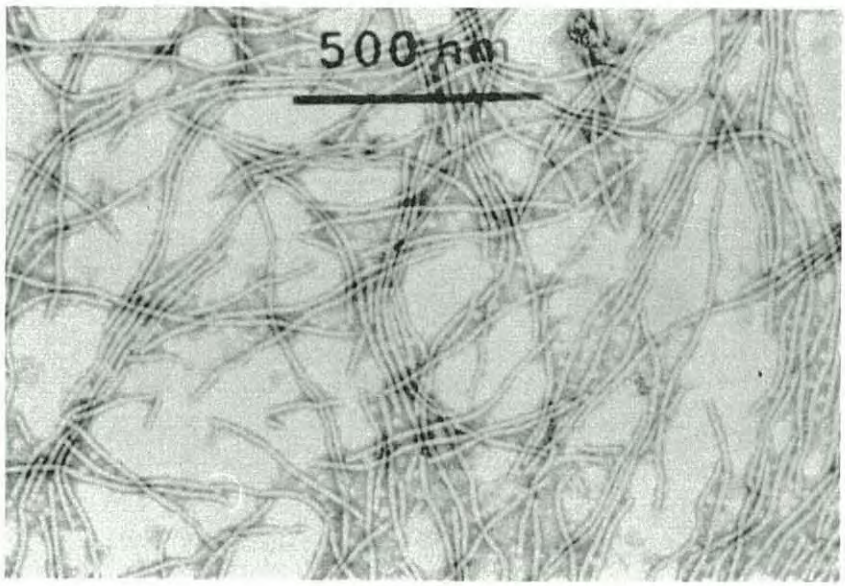
The SDV-Ap, SDV-As and Ap-45 (an SDV isolate from Gilroy, California which is transmitted specifically by *A. pisum*) were compared serologically using DAS-ELISA.

5.3 Results

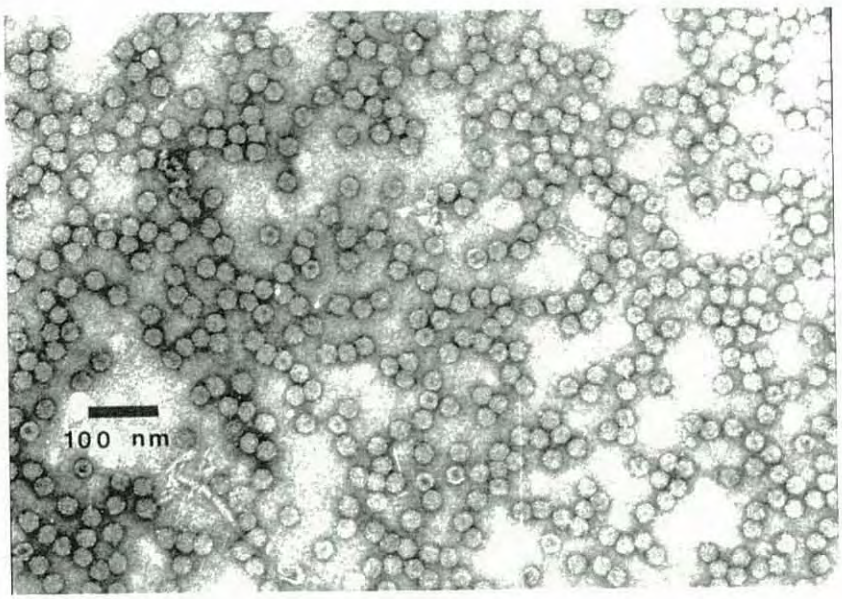
Properties of purified viruses

Purified preparations of BYMV-F and CYVV-D were colourless and opalescent. The purified viruses showed an ultraviolet absorption curve typical for potyviruses with a maximum at 260nm and minimum at 245-247nm. The $A_{260/280nm}$ varied from 1.21-1.35, with an average of 1.26 for BYMV-F and 1.29 for CYVV-D. Purified preparations of both isolates formed single light scattering and ultraviolet absorption bands in sucrose density gradients. The virions were flexuous rods with a length of 700-750 x *c.*12nm (Figure 5.1). On the basis of an extinction coefficient 2.4 (OD 260) the yields of purified BYMV-F and CYVV-D were estimated to be 0.8 - 2.5 and 0.6 - 1.2 mg/100g of fresh infected leaf tissue respectively.

SDV-Ap purified had isometric particles *c.* 27nm in diameter (Figure 5.1). Virus yields were low, typical of luteoviruses and were generally between 0.3 and 0.8mg/kg of tissue. Absorbance spectra of purified preparations were typical of nucleoprotein preparations with a maximum at 260nm and a minimum at 242nm. $A_{260/280nm}$ ratios in purified virus preparations had an average value of 1.83. The virus formed a single ultraviolet absorbing zone in sucrose density gradients (Figure 5.2). More virus was recovered



(a)



(b)

FIGURE 5.1

Electron micrographs of the purified virus preparations stained with 2% uranyl acetate:

(a) BYMV-F potyvirus (b) SDV-Ap luteovirus.

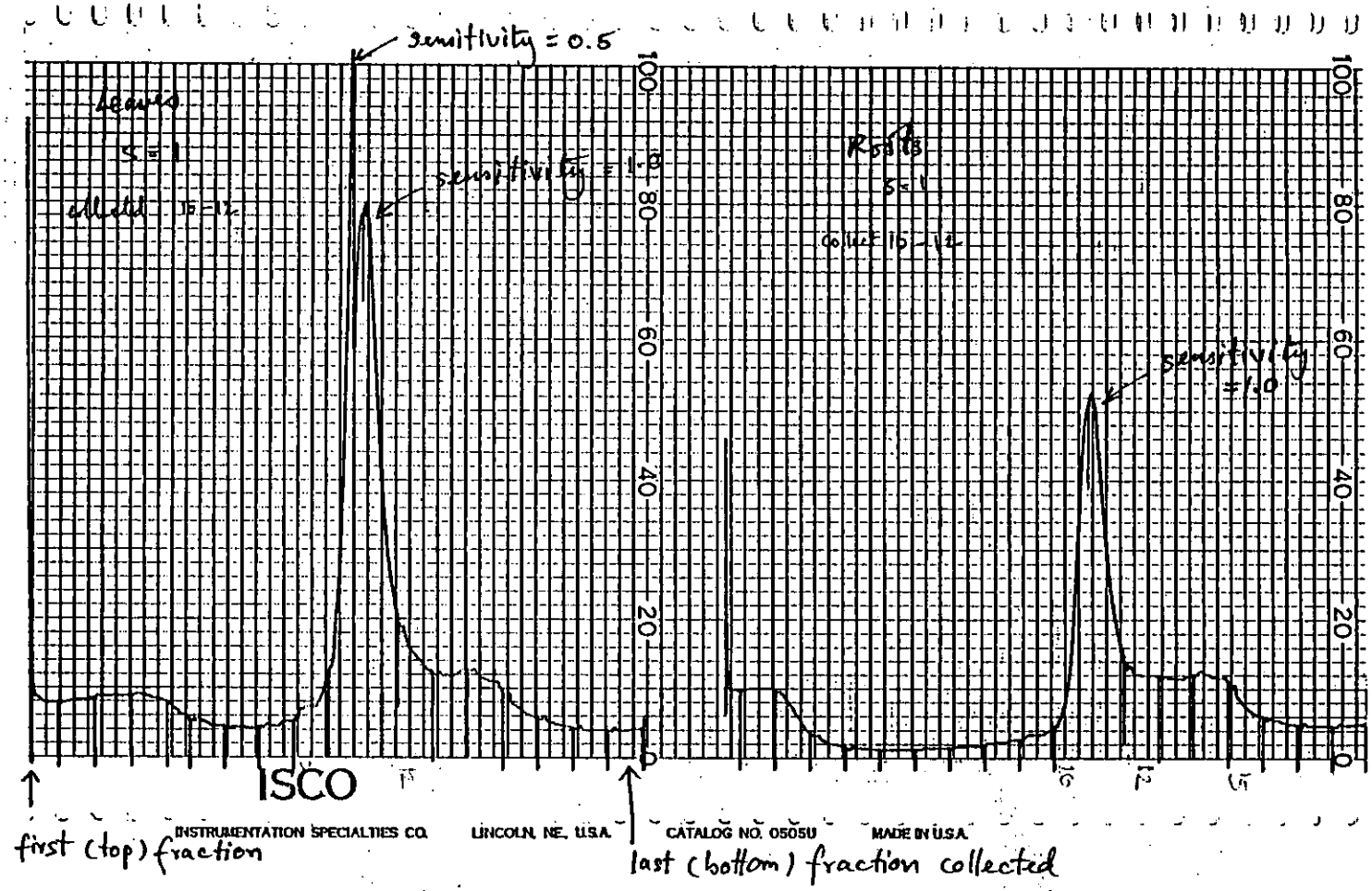


FIGURE 5.2 Absorption spectra of sucrose density gradient columns from purification of SDV-Ap using an ISCO UA5 density gradient fractionator.

when tissues were incubated with enzyme (Celluclast) for 16 hours than when they were incubated for only six hours (Table 5.2). The yield of SDV-Ap purified from infected roots (588µg/kg) was less than that from infected leaves (Table 5.2). Infected tissue (leaves and roots) incubated with Celluclast for 16 hours was used as a virus source to produce antiserum.

Serological tests

The optimal concentration of purified IgG for coating of BYMV-F, CYVV-D and SDV-Ap was 1.11µg/ml (Figure 5.3). The results in Figure 5.4 showed that the optimal dilutions of conjugated purified IgG were approximately 1:1500, 1:500 and 1:750 for BYMV-F, CYVV-D and SDV-Ap respectively. The best working dilutions of crude sap extracts used in ELISA tests was 1:10 for BYMV-F and 1:4 for CYVV-D and SDV-Ap.

The results from the indirect ELISA tests (Figure 5.5) indicated that the titre of BYMV-F antiserum was higher than those of CYVV-D and SDV-Ap from similar bleeds. The titres of each virus antiserum generally increased with time. The IgGs for ELISA tests were purified from those antisera that gave the highest titre. For example, IgG of SDV-Ap was purified from the 7th and 8th bleeds.

The serological relationships of the two pairs of potyviruses were further investigated using polyclonal and monoclonal antibodies in DAS-ELISA. The results indicated that BYMV and CYVV isolates were clearly distinct (Table 5.3). In addition, the results of IEM tests indicated that particles of the two CYVV isolates were trapped by CYVV-D antiserum at much higher levels than those of the BYMV isolates and vice versa. Average numbers of trapped virus particles per EM field by BYMV-F coated grids (10 fields per sample) were

TABLE 5.2

Comparison of absorption ratios and yields of SDV-Ap preparations using different periods of incubation with Celluclast in extracts from shoots and roots.

Treatment	$A_{260/280nm}$	Yield (µg/kg of tissue)
Incubation		
6 hours	1.64	345
16 hours	1.82	371
Virus source		
leaves	1.83	717
roots	1.72	588

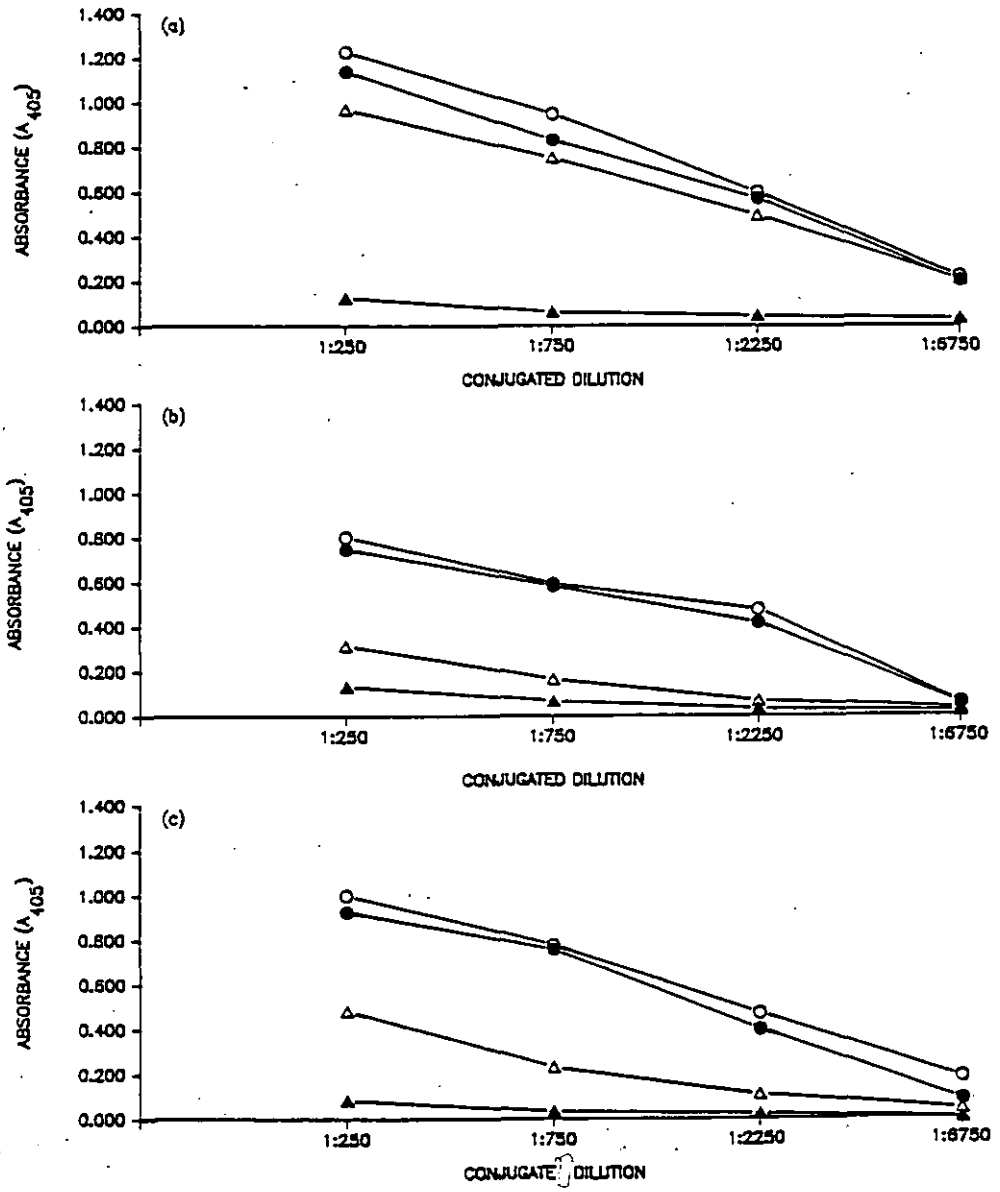


FIGURE 5.3

Relationships between IgG dilutions [0.37 (△—△), 1.11 (●—●) and 3.33 (o—o)] and optical densities for various dilutions of conjugates. The crude sap extracts from infected plants of BYMV-F (a), CYVV-D (b) and SDV-Ap (c) were prepared by grinding tissue in extracting buffer at 1:11.1, 1:3.7 and 1:3.7, w:v respectively. (▲—▲) signifies extracts from healthy plants.

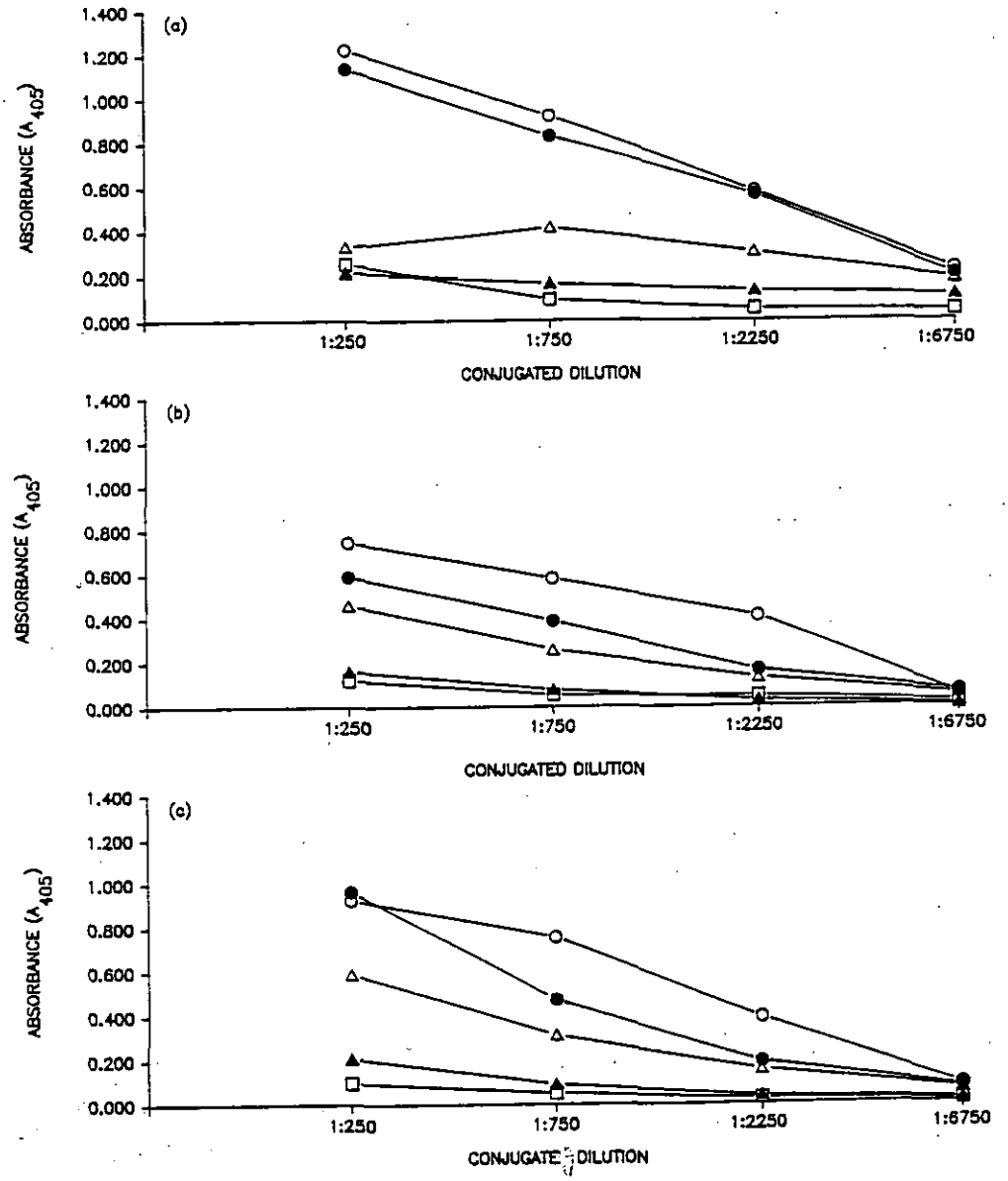


FIGURE 5.4

Relationships between crude sap dilutions 1:3.70 (o—o), 1:11.11 (●—●), 1:33.33 (△—△) and 1:100 (▲—▲) and optical densities for various dilutions of the conjugated immunoglobulins. The plates were coated with the purified IgG's of BYMV-F (a), CYVV-D (b) and SDV-Ap (c) at 1.11µg/ml in carbornate coating buffer. (□—□) signifies extracts from healthy plants.

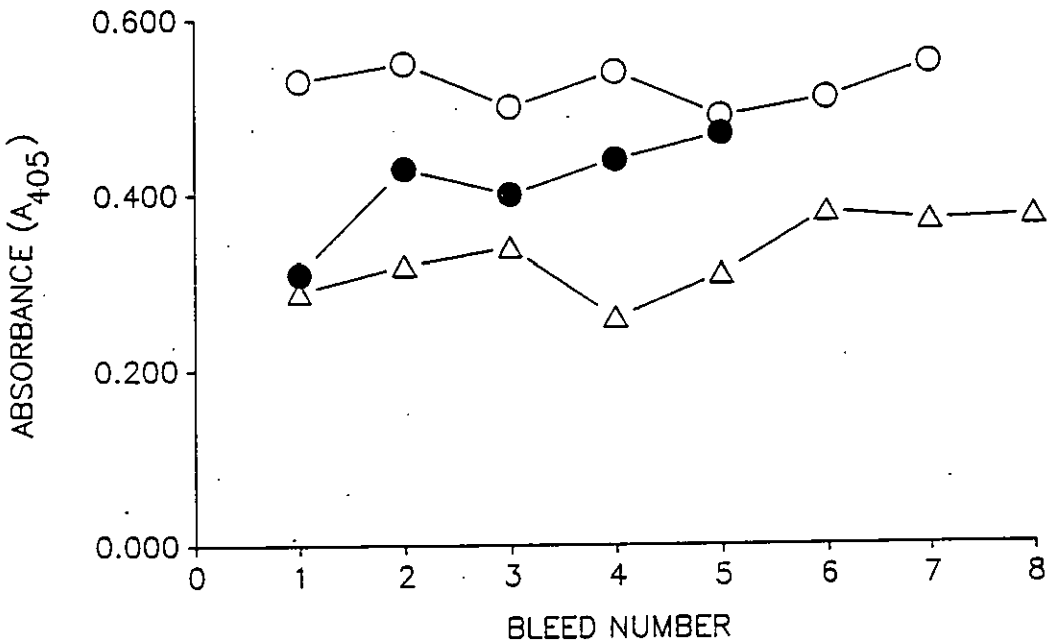


FIGURE 5.5

Absorbance values (A_{405}) from indirect ELISA tests using sera from various bleeds of rabbits that were injected with purified preparations of BYMV-F (o—o), CYVV-D (●—●) and SDV-Ap (△—△).

TABLE 5.3

Absorbance values (A_{405}) from double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) on extracts from plants infected with pairs of isolates of bean yellow mosaic virus (BYMV) and clover yellow vein virus (CYVV).

Virus isolate	Antiserum	
	BYMV-F	CYVV-D
BYMV-F	0.62	0.18
BYMV-K	0.65	0.12
CYVV-B	0.15	0.41
CYVV-D	0.13	0.54
Healthy	0.05	0.15

35.4, 30.1, 16.3 and 14.6 for BYMV-F, BYMV-K, CYVV-B and CYVV-D respectively. On the other hand, CYVV-D antiserum coated grids trapped 13.8, 11.2, 22.7 and 26.5 particles per field of BYMV-F, BYMV-K, CYVV-B and CYVV-D. Similarly the two BYMV antisera from the U.K. trapped BYMV-F and BYMV-K particles in large numbers on electron microscope grids compared to CYVV-B and CYVV-D whereas the two CYVV antisera from the U.K. trapped many particles of CYVV-B and CYVV-D but very few particles from extracts of plants infected with the two BYMV isolates (Table 5.4). However, the isolates of BYMV and CYVV could not confidently be distinguished using the potyvirus monoclonal antibody provided by Dr. Shukla (Figure 5.6). Similarly a potyvirus monoclonal antibody developed by Jordan and Hammond (1986) to an internal sub-unit epitope and now marketed commercially by Agdia Inc. detected all four isolates with ease.

Tests by DAS-ELISA with the luteovirus antisera to BWYV, LYV, SDV-Ap and SDV-As and their homologous viruses indicated that the two SDV isolates were serologically similar but clearly different from BWYV and LYV (Table 5.5). The concentrations of serologically detectable virus in sap extracts from plants infected with SDV-As were much higher than those in extracts from plants inoculated with SDV-Ap or Ap-45 irrespective of whether the coating and conjugated globulins used in the tests were prepared from antisera to either SDV-Ap, SDV-As or Ap-45 (Table 5.6). It was therefore impossible to use the SDV antisera to confidently distinguish between the different aphid specific isolates of SDV in field samples. In addition, tests done in conjunction with Dr. P. M. Waterhouse established that a number of cDNA probes produced to SDV-As (Waterhouse *et al.*, 1986) failed to distinguish between the SDV-As and SDV-Ap isolates.

TABLE 5.4

Relative numbers of potyvirus particles from crude sap extracts of plants infected with BYMV-F, BYMV-K, CYVV-B and CYVV-D by two BYMV antisera and two CYVV antisera provided by the Glasshouse Crops Research Institute.

Virus isolate	Coated Antiserum			
	BYMV 226	BYMV 307	CYVV 87	CYVV 94
BYMV-F	15.8*	5.1	1.8	0.7
BYMV-K	18.9	6.3	1.6	0.9
CYVV-B	1.3	0.2	28.5	15.2
CYVV-D	0.9	0.1	29.0	12.8

* Mean numbers of particles per field based on counts of ten fields on each of four grids.

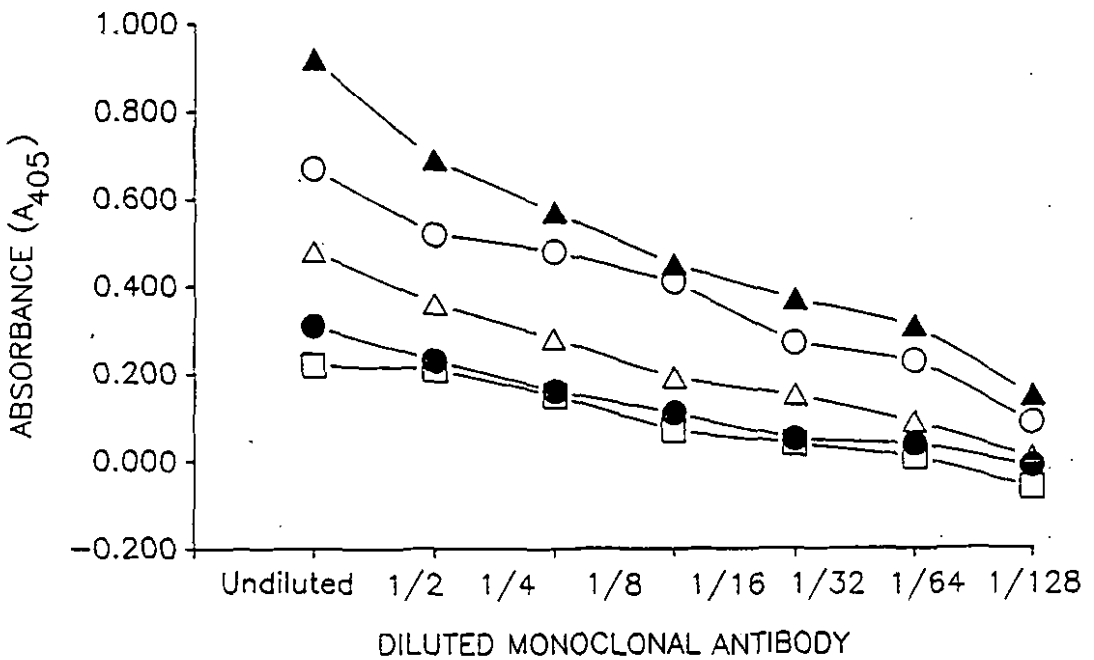


FIGURE 5.6

Absorbance values (A_{405}) from indirect ELISA tests using monoclonal antibody (DS-6-Fusion-1-16-B) bound to BYMV-F (o—o), BYMV-K (●—●), CYVV-B (△—△), CYVV-D (▲—▲) and healthy tissues (□—□).

TABLE 5.5

Absorbance values (A_{405}) from DAS-ELISA tests on preparations of bean leaf roll (legume yellows, LYV), beet western yellows (BWYV) and soybean dwarf (SDV-Ap and SDV-As) viruses using IgG's and conjugates prepared from polyclonal antisera produced against the four viruses.

Antiserum	Virus				
	BWYV	LYV	SDV-Ap	SDV-As	Healthy
BWYV	1.65	0.02	0.01	-0.02	0.02
LYV	0.22	0.77	0.06	0.07	0.10
SDV-Ap	0.01	0.06	0.65	0.93	0.02
SDV-As	0.02	0.06	0.38	1.95	0.01

TABLE 5.6

Absorbance values (A405) from DAS-ELISA tests on preparations of vector specific isolates of soybean dwarf virus (Ap-45, SDV-Ap and SDV-As) using IgG's and conjugates prepared from polyclonal antisera produced against the three virus isolates.

Coating antibody	Conjugate antibody	Virus isolate			
		Ap-45	SDV-Ap	SDV-As	Healthy
Ap-45	Ap-45	0.30*	0.24	0.96	0.10
	SDV-Ap	0.19	0.26	0.79	0.06
	SDV-As	0.21	0.26	0.95	0.07
SDV-Ap	Ap-45	0.31	0.24	0.91	0.09
	SDV-Ap	0.26	0.46	0.67	0.06
	SDV-As	0.21	0.23	0.87	0.06
SDV-As	Ap-45	0.33	0.24	0.91	0.09
	SDV-Ap	0.20	0.24	0.65	0.05
	SDV-As	0.24	0.20	0.91	0.07

* Mean absorbance values of four replicate wells.

The readings were taken 30 minutes after the substrate was added.

5.4 Discussion

Aggregation of virus particles during purification is a limiting factor in obtaining high yields of potyviruses. Two types of aggregation have been suggested by Choi *et al.* (1977), hydrophobic bonding of virus particles with host materials and inter-particle binding. Consequently, the composition of the extracting buffer was particularly important in protecting against such aggregation. Use of high molarity extracting buffers plus additives such as Na-DIECA, EDTA and urea have been useful in inhibiting aggregation of potyvirus particles (Damirdagh & Shepherd, 1970; Vovlas & Russo, 1978). The inclusion of urea and 2-ME in resuspending buffers have been reported to protect against the aggregation of virus particles during sucrose density gradient centrifugation (Uyeda *et al.*, 1975; Vovlas & Russo, 1978). The methods used here resulted in good yields of BYMV-F and CYVV-D with a high degree of purity.

The yields of luteoviruses are affected by the propagation host and virus strain, but it is especially important to develop an effective method of extracting the particles from the phloem tissue. Grinding in liquid nitrogen (Rochow *et al.*, 1971; Hewings *et al.*, 1986), a fruit press (Rowhani & Stace-Smith, 1979), or grinding *first in a blender and then in a VirTis homogenizer* (Ashby & Huttinga, 1978) have been used with some success to extract luteoviruses. Enzyme preparations (cellulases, pectinases) added to the extracting buffer are also helpful in increasing virus yields (Waterhouse & Helmes, 1984). More virus was recovered when infected tissue was incubated with enzyme for 16 hours than for 6 hours. Due to the long incubation period it was necessary to add NaN_3 (0.02%) to the extracting buffer to protect from bacterial contamination

(Waterhouse & Murant, 1981).

Serology has been a major criterion for identification and separation of potyviruses (Hollings & Brunts, 1981). DAS-ELISA was used to study serological relations among isolates quantitatively because that method distinguished between BYMV subgroups better than indirect ELISA (Lawson *et al.*, 1985). The direct ELISA test (DAS-ELISA) was able to differentiate between BYMV and CYVV isolates but strains of BYMV and CYVV were not distinguished. The monoclonal antibodies to potyvirus isolates provided by Dr. Shukla and available from North America were not useful in differentiating between the two groups of potyviruses.

The results from DAS-ELISA tests on serological relationships between BLRV(LYV), BWYV, SDV-Ap and SDV-As confirmed earlier reports on interrelationships within the luteovirus group (Ashby & Huttinga, 1978; Johnstone *et al.*, 1989; Waterhouse *et al.*, 1988). SDV-Ap and SDV-As were serologically related but they were distinct from LYV and BWYV. It was of interest that the SDV isolates that had different species specific vectors could not be distinguished serologically even though the coat protein that confers serological specificity is believed to also control vector specificity by allowing transport across the gut wall and through the salivary glands of the aphids (Gildow & Rochow, 1980). In the barley yellow dwarf system, there are clear serological differences between members of the group that are closely correlated with variations in vector specificity (Rochow & Carmichael, 1979). Work is in progress to confirm presumptive evidence that the read-through translational product of the coat protein gene controls vector specificity of the vector specific SDV luteovirus isolates whereas the *c.* 22K coat protein gene itself encodes for the viral sub-units (Veidt *et al.*,

1988). The data in Table 5.6 indicated that concentration of SDV-As in the host plants was higher than that of SDV-Ap or Ap-45.

CHAPTER 7

General Conclusions

7.1 Over-View of Results

The investigations reported here have contributed to the understanding of luteovirus and potyvirus disease problems of legumes in Tasmania. They indicate the importance of environmental influences and selection pressures on the distribution and incidence of virus disease problems. The complexities of the interactions between the environment, different virus biotypes, their plant hosts and their aphid vectors were clearly demonstrated. The development of detection techniques and the recognition of virus variants reported here should help towards better approaches to the control of these diseases in the future. The general principles developed in these studies should have world-wide applicability.

Set out below are 25 findings from this study that are considered to be of major importance with respect to plant virus epidemiology and control, and which highlight the complex nature of environmental influences on interactions between plant viruses, their host plants, and aphid vectors.

* viruses found infecting subterranean clover and faba bean in Tasmania were alfalfa mosaic, bean yellow mosaic, clover yellow vein, cucumber mosaic, soybean dwarf, subterranean clover mottle and subteranean stunt viruses.

* the incidence of these viruses was very variable between sites and between years.

* variations in virus incidence partly reflected levels of

aphid flight activity of the vector species and these were generally unpredictable although there were fairly consistent seasonal fluctuations in activity.

* settling of aphid alatae on crops and pastures was significantly influenced by crop age, ground cover and plant density.

* the most prevalent viruses were BYMV and SDV.

* different isolates of BYMV and SDV differed with respect to their vector specificities and host ranges.

* variability in the biological properties of virus isolates was common and partly reflected differences in cropping patterns and pasture composition between different regions of the State.

* numbers of various species of alate aphids trapped at two locations only twenty kilometres apart differed and were markedly influenced by patterns of cropping nearby, thus demonstrating that aphid activity was not consistent even on a regional basis.

* this study provided the first record of *Brachycaudus rumexicolens* in Australia.

* under Tasmanian conditions, autumn sowing of broad beans not only minimised losses due to infection with BYMV and SDV but also maximised potential yields of the crop.

* the non-colonizing aphid species *Rhopalosiphum padi* seemed to be a significant factor in the spread of the non-persistent BYMV isolates when this species was present near faba bean plots in large numbers.

* yield losses of broad beans were greatest when the plants became infected before flowering commenced.

* seed-borne virus transmission has a vital role in the ecology of the non-persistent BYMV because low numbers of infection foci can lead to extensive crop losses.

* broad bean crops should be positioned as far away as possible from, and up-wind of, lucerne stands which are a host of *Acyrthosiphon pisum* and an over-wintering and over-summering source of SDV-Ap (in south-eastern Tasmania).

* the incidence of virus infections in legume pastures and field crops are controlled by complex interactions between environmental influences on the viruses, their host plants and their aphid vectors.

* the pattern of BYMV spread was contagious and mostly originated from infection sources within crops whereas SDV spread into the plots from outside.

* the relative abundance of different vector-specific isolates of SDV in Tasmania differed between the north-western and south-eastern regions of the State and was correlated with the relative abundance of different reservoir host plants of the viruses and their aphid vectors.

* climatic variables such as rainfall can alter the relative abundance of different SDV isolates in a region due to their influence on the prevalence of different plant species serving as reservoirs and hosts of various aphid vector species.

* pairs of BYMV and CYVV potyviruses that varied in vector specifity were not distinguishable serologically when compared using monoclonal and polyclonal antibodies to potyvirus coat proteins.

* differences in vector specificity of various potyvirus isolates reflected differences in the HC's incited in the plants they infected.

* dependent transmission phenomena with the potyvirus isolates was consistent with differences in HC properties but experiments to confirm this conclusively were unsuccessful because none of the isolates was ever transmitted after allowing aphids to feed on virus and HC preparations through Parafilm membranes.

* SDV luteovirus isolates that varied in vector specificity could not be distinguished serologically or by nucleic acid hybridization analysis, and in this respect differed from the barley yellow dwarf luteovirus systems reported from Europe and North America and from synthetic mixtures of carrot red leaf virus and other luteoviruses (Waterhouse & Murant, 1983).

* differences in vector specificity of the SDV isolates were also associated with some differences in host range and thus permitted experimentation on dependent transmission from mixed infections.

* SDV-Ap was transmitted by *A. solani* from mixed infections as was SDV-As by *A. pisum*.

* dependent transmission phenomena enhance the opportunities for survival of luteoviruses and potyviruses by extending their host ranges and modes of transmission.

7.2 Conference Presentations

The following papers and posters were presented at national and international conferences during the course of these studies.

Srithongchai, W. (1986). Aphid vector specificity of bean yellow mosaic and clover yellow vein potyviruses. *Proceedings of the Cradle Mountain Virology Workshop, Cradle Valley, Tasmania, 11-14 November 1986.*

Srithongchai, W. (1988). The development since 1980 and properties

of soybean dwarf virus in Tasmania transmitted specifically by *Acyrthosiphon pisum*. *Proceedings of a National Aphid Workship, CSIRO Division of Entomology, Canberra, 23-24 August 1988.*

Srithongchai, W. (1988). Transmission by aphids of five potyviruses isolated from legumes. *Proceedings of a National Aphid Workshop, CSIRO Division of Entomology, Canberra, 23-24 August 1988.*

Srithongchai, W. & Johnstone, G. R. (1987). A change in the vector specificity of commonly occurring soybean dwarf virus isolates in Tasmania. *Proceedings of the Sixth Australian Plant Pathology Conference, Adelaide, South Australia, 11-15 May 1987.* p 108. (Poster)

Srithongchai, W. & Johnstone, G. R. (1988). A change in the vector specificity of commonly occurring soybean dwarf virus isolates in Tasmania. *Proceedings of the 5th International Congress of Plant Pathology, Kyoto, Japan, 20-27 August 1988.* p. 51. (Poster)

Srithongchai, W. & Johnstone, G. R. (1988). Host ranges and transmission by aphids of five potyviruses isolated from legumes in Australia. *Proceedings of the 11th Meeting of the International Working Group on Legume Viruses, Kurashiki, Japan, 29-30 August 1988.* p. 13.

Srithongchai, W. & Johnstone, G. R. (1988). Occurrence and properties of soybean dwarf viruses in Tasmania. *Proceedings of the 11th Meeting of the International Working Group on Legume Viruses, Kurashiki, Japan, 29-30 August 1988.* p. 10.

7.3 Research Papers to be published from this Study

The following papers documenting parts of the work reported in this thesis are in the course of preparation for submission to refereed journals.

Johnstone, G. R. & Srithongchai, W. (1990). Dependent transmission of viruses by aphids from mixed infections. I. Aphid specific variants of soybean dwarf luteoviruses. (In preparation for Australian Journal of Agricultural Research.)

Johnstone, G. R. & Srithongchai, W. (1990). Variation in the occurrence of different vector specific isolates of soybean dwarf virus between the north-western and south-eastern regions of Tasmania. (In preparation for Australian Journal of Agricultural Research.)

Johnstone, G. R., Wakamatsu, K., Srithongchai, W. & Kojima, M. (1990). Host range and transmission of the agent causing subterranean clover stunt and its similarities to milk vetch dwarf virus. (In preparation for Australasian Plant Pathology.)

Srithongchai, W. & Johnstone, G. R. (1990). Aphid flight activity at two sites in south-eastern Tasmania over three successive years. (In preparation for Australian Journal of Zoology.)

Srithongchai, W. & Johnstone, G. R. (1990). Dependent transmission of viruses by aphids from mixed infection. II. Aphid specific variants of bean yellow mosaic and clover yellow vein potyviruses. (In preparation for Australian Journal of Agricultural Research.)

Srithongchai, W. & Johnstone, G. R. (1990). Effects of soybean dwarf luteovirus and bean yellow mosaic potyviruses on *Vicia faba* production in south-eastern Tasmania. (In preparation

for Australian Journal of Experimental Agriculture.)

Srithongchai, W. & Johnstone, G. R. (1990). Occurrence and spread of luteoviruses and potyviruses in *Vicia faba* crops in south-eastern Tasmania over three successive years. (In preparation for Annals of Applied Biology.)

Srithongchai, W. & Johnstone, G. R. (1990). Occurrence of viruses in Tasmanian subterranean clover pastures. *Australasian Plant Pathology* (submitted).

Williams, M. A., Johnstone, G. R. & Srithongchai, W. (1990). Notes on *Brachycaudus rumexicolens* (Patch) (Homoptera : Aphididae) in Tasmania, newly recorded from Australia. (In preparation for Journal of the Australian Entomological Society.)

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Abu-Samah, N. & Randles, J. W. (1981). A composition of nucleotide sequence homologies of three isolates of bean yellow mosaic virus and their relationship to other potyviruses. *Virology* 110 : 436-444.

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APPENDICES

APPENDIX 1

Dates of various events associated with the 1986-1987 field studies.

Date of	Month sown		
	April	September	November
Sowing	24 Apr	23 Sep	18 Nov
Seedling emergence	25 May	15 Oct	10 Dec
First insecticide application	26 May	16 Oct	11 Dec
Virus inoculation	10 Jun	24 Oct	18 Dec
First date of observation	25 Jun	7 Nov	30 Dec
Harvest	2 Dec	2 Jan	17 Feb

APPENDIX 2

Detail of *Vicia faba* crops record in 1987-1988.

Date of:	Month sown		
	May	September	November
Sowing	8 May	9 Sep	8 Nov
Seedling emergence	31 May	25 Sep	30 Nov
First insecticide application	2 Jun	27 Sep	2 Dec
Virus inoculation	5 Jun	3 Oct	8 Dec
First date of observation	17 Jun	15 Oct	22 Dec
Harvest - green beans	11 Nov	28 Dec	2 Feb
Harvest - dried beans	21 Jan	14 Feb	30 Mar

APPENDIX 3

Alate aphid catches* of the major aphid species in Moericke trays at Cambridge and Sandford from 1986 to 1988.

Aphid species	Site	Jan 86 to Jun 86	Jul 86 to Dec 86	Jan 87 to Jun 87	Jul 87 to Dec 87	Jan 88 to Jun 88	Jul 88 to Dec 88	Mean	S.E.
A. kondoi	Cambridge	0	4	0	1	1	2	1.3	0.6
	Sandford	0	1	0	0	0	2	0.5	0.3
A. pisum	Cambridge	5	36	6	4	1	8	10.0	5.3
	Sandford	10	34	6	3	1	10	10.7	4.9
A. craccivora	Cambridge	11	36	22	6	30	12	19.5	4.8
	Sandford	2	5	9	3	20	11	8.3	2.7
A. solani	Cambridge	1	4	0	0	0	1	1.0	0.6
	Sandford	0	0	0	0	0	1	0.2	0.2
B. rumexicolens	Cambridge	1	288	17	53	369	34	127.0	65.0
	Sandford	0	78	57	14	25	13	31.2	12.2
B. brassicae	Cambridge	106	41	31	205	991	6	230.0	155.0
	Sandford	37	5	14	3	10	2	11.8	5.4
C. aegopodii	Cambridge	249	7	470	614	132	62	255.7	98.2
	Sandford	1	2	5	2	4	0	2.3	0.8

APPENDIX 3 (continued)

Aphid species	Site	Jan 86 to Jun 86	Jul 86 to Dec 86	Jan 87 to Jun 87	Jul 87 to Dec 87	Jan 88 to Jun 88	Jul 88 to Dec 88	Mean	S.E.
D. aucupariae	Cambridge	398	32	104	55	27	14	105.0	60.0
	Sandford	172	25	730	316	213	51	251.2	105.3
M. euphorbiae	Cambridge	2	10	0	0	1	1	2.3	1.6
	Sandford	0	5	0	1	2	1	1.5	0.8
M. persicae	Cambridge	10	8	5	1	12	2	6.3	1.8
	Sandford	1	1	4	2	7	1	2.7	1.0
R. padi	Cambridge	21	61	18	20	5	19	24.0	7.8
	Sandford	12	32	9	8	1	14	12.7	4.3
Total aphids	Cambridge	837	577	685	1017	1828	169	852.2	227.3
	Sandford	244	217	837	366	299	117	346.7	103.8

*Mean catch per tray per week.

A. kondoi = *Acyrthosiphon kondoi*
A. pisum = *Acyrthosiphon pisum*
A. craccivora = *Aphis craccivora*
A. solani = *Aulacorthum solani*
B. rumexicolens = *Brachycaudus rumexicolens*
B. brassicae = *Brevicoryne brassicae*
C. aegopodii = *Cavariella aegopodii*
D. aucupariae = *Dysaphis aucupariae*
M. euphorbiae = *Macrosiphum euphorbiae*
M. persicae = *Myzus persicae*
R. padi = *Rhopalosiphum padi*

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